Azithromycin in periodontal therapy: pharmacokinetic and mechanistic investigations

DISSERTATION

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Abstract

Bacterial plaque is a primary etiological factor of periodontitis, and a specific group of pathogens is strongly associated with this destructive inflammatory entity. Although conventional periodontal scaling and root planing usually halts the disease process, the loss of bone and periodontal attachment continues in some patients. Aggressive and recurrent forms of periodontitis are associated with persistent infection by Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis. These pathogens can invade gingival epithelium and fibroblasts, allowing them to evade the host immune system and re-colonize pockets after debridement. Although the use of adjunctive systemic antibiotics to treat invasive infection by these pathogens is recommended, an agreement on the most effective regimen has not been reached.

Although azithromycin was developed more than 20 years ago, it is not commonly used in periodontal therapy. Its favorable properties include inhibition of a broad spectrum of periodontal pathogens, a high volume of distribution, a long half-life, and relatively low incidence of side effects. Studies have indicated that human cells take up azithromycin and active transport may be involved with this process. In this project, we hypothesize that gingival epithelial cells, fibroblasts, and neutrophils possess active transporters that accumulate azithromycin. These transporters may enhance and sustain levels of azithromycin in cellular compartments of gingiva and in gingival crevicular fluid. The accumulated azithromycin may facilitate elimination of intracellular periodontal pathogens. The chapters of this dissertation are 1) to study the mechanism by which azithromycin is transported by cells in the gingiva, and 2) to evaluate the benefits of this transport system with regards to pharmacokinetics and antimicrobial activity against intracellular A. actinomycetemcomitans and P. gingivalis. The characterization of azithromycin transport was conducted in human cells, using radiolabeled azithromycin. To study the mechanism behind the transport, agents with similar
molecular features were added to the assays and their effects on azithromycin transport were determined. To study the pharmacological benefits of azithromycin transport, human subjects with clinically healthy periodontal tissues were recruited. They were first standardized in their oral hygiene and gingival health by full-mouth debridement and oral hygiene instruction. Following systemic administration of azithromycin, serum and gingival crevicular fluid were sampled and the content of azithromycin was determined by agar diffusion bioassays with *Kocuria rhizophila* as the indicator microorganism. The intracellular antimicrobial activity of azithromycin was evaluated in gingival epithelial cells, fibroblasts, and neutrophils. Periodontal pathogens were first internalized in these cells, and the surviving colony-forming units after antibiotic treatment were measured by growing the cellular lysates on appropriate agar plates.

Our data suggest that gingival epithelial cells, fibroblasts, and neutrophils possess saturable, concentrative active transport systems for azithromycin that are shared with organic cations. The transport systems lead to high degrees of intracellular accumulation in gingival epithelial cells, fibroblasts, and neutrophils, with cellular/extracellular concentration ratios of ~20, ~11, and ~5, respectively. Azithromycin concentration in gingival crevicular fluid is significantly higher and more sustained than that in serum over 2 weeks. In inflamed gingiva, inflammatory infiltrates including neutrophils help to deliver azithromycin, leading to an increased perfusion of azithromycin into the gingival crevice. Accumulation of azithromycin in human gingival epithelial cells, fibroblasts, and neutrophils enhances the elimination of invasive *A. actinomycetemcomitans* and *P. gingivalis*. Compared to antibiotics that are not concentrated by host cells, azithromycin exhibits either equivalent or more potent antimicrobial activities. Competitive inhibitors of azithromycin transport significantly reduce the intracellular content of azithromycin and attenuate its antimicrobial effects. Overall, this project provides a rational basis for adjunctive use of azithromycin in periodontal therapy.
Dedication

Dedicated to my parents Hsi-Ming and Kwei-Yu, my brother Pin-Heng, my sister Ping-Hsiu, and my girl friend Mei-Hsuan Huang.
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**Fields of Study**

Major Field: Oral Biology
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Chapter 1

Should antibiotics be prescribed to treat chronic periodontitis?

1.1 Abstract

Although scaling and root planing is a cost-effective approach for initial treatment of chronic periodontitis, it fails to eliminate subgingival pathogens and halt progressive attachment loss in some patients. Adjunctive use of systemic antibiotics immediately after completion of scaling and root planing can enhance the degree of clinical attachment gain and probing depth reduction provided by nonsurgical periodontal treatment. This article discusses the rationale for prescribing adjunctive antibiotics, reviews the evidence for their effectiveness, and outlines practical issues that should be considered before prescribing antibiotics to treat chronic periodontitis.

1.2 Introduction

Periodontitis is a chronic inflammatory disease that leads to destruction of the supporting tissues of teeth and, if left untreated, tooth loss. Severe periodontitis was the world’s sixth-most prevalent condition in 2010; its age-standardized prevalence between 1990 and 2010 among all countries was 11.2%. Consistent with this estimate, a study based on data from the 2009 and 2010 National Health and Nutrition Examination Survey cycle reported prevalence rates of 8.7%, 30.0%, and 8.5% for mild, moderate, and severe periodontitis, respectively, in the United States.

Studies from the past 3 decades have revealed that only a small subset of microorganisms from among the hundreds of species found in the oral cavity is highly associated with periodontitis. Although specific biofilm-producing bacterial pa-
thogens and other cooperative species are required, bacteria alone are not sufficient to induce periodontitis. The host immune-inflammatory response is a determinant of susceptibility to periodontitis and is responsible for most of the periodontal tissue destruction. During persistent bacterial infection and prolonged homeostatic imbalance, cytokines and enzymes released by host leukocytes mediate destruction of periodontal connective tissue and bone. Systemic diseases (eg, diabetes), immune dysfunction, and environmental factors (eg, smoking) can also contribute to disruption of the homeostatic balance. The goal of periodontal therapy is to preserve the natural dentition in stability, comfort, and function by eliminating pathologic biofilm and resolving inflammation.

Microbial complexes in subgingival biofilm have been recently characterized using molecular techniques. Individual species in these complexes have been assigned using a color-coded system that reflects community ordination and cluster analysis. The red complex, consisting of Tannerella forsythia, Porphyromonas gingivalis, and Treponema denticola, is strongly associated with severe chronic periodontitis. The orange complex, which includes Prevotella intermedia, Fusobacterium nucleatum, Campylobacter rectus, and Peptostreptococcus micros, is closely associated with the red complex. The green complex includes Aggregatibacter actinomycetemcomitans, which has a strong association with aggressive periodontitis and a less frequent association with chronic periodontitis. Porphyromonas gingivalis, A. actinomycetemcomitans and other pathogens possess virulence factors that can overcome the host response and damage periodontal tissues.

Porphyromonas gingivalis, A. actinomycetemcomitans, and Prevotella intermedia are capable of invading the epithelium of periodontal pockets, which protects them from elimination by the host response, making them exceptionally difficult to eliminate by conventional periodontal scaling and root planing (SRP). Persistent infections by these bacteria are frequently associated with progressive chronic periodontitis. Another limitation of SRP is that it is not effective in removing bacteria from deep pockets, furcations, dentinal tubules, and other subgingival sites where access is poor. The difficulties associated with eliminating bacteria that have colonized the soft tissue wall of the pocket and other inaccessible areas provide a rationale for incorporating systemic
antibiotics into the treatment of periodontitis.

A broad range of systemic antibiotics has been used to treat chronic periodontitis. The pharmacokinetic and antimicrobial properties of the agents used most commonly are presented in Table 1 and information on dosage is detailed in Table 2. In general, amoxicillin, metronidazole, azithromycin, tetracycline, and doxycycline are capable of attaining levels that can effectively inhibit periodontal pathogens when they are growing as single (planktonic) cells in a periodontal pocket or the soft tissue wall of a pocket. The exception is metronidazole, which exhibits relatively poor activity against *A. actinomycetemcomitans* at typical in vivo concentrations. However, it is important to remember that subgingival bacteria live in a biofilm, not as single cells. Bacteria growing in a biofilm are substantially more difficult to inhibit with antibiotics. For this reason, antibiotics should only be used to treat periodontitis in patients who have already had their subgingival biofilm disrupted by SRP.

Unlike the other agents in Table 1, azithromycin and doxycycline have relatively long half-lives and are normally administered in a single daily dose. Azithromycin and tetracycline compounds are actively taken up and concentrated inside oral epithelial cells, whereas amoxicillin and metronidazole enter cells by passive diffusion. This property may be useful for targeting periodontal pathogens that have invaded the pocket epithelium. When cultured gingival epithelial cells infected with *A. actinomycetemcomitans* are incubated with physiologic concentrations of azithromycin (8 mg/mL), azithromycin accumulates inside the epithelial cells at levels that kill more than 80% of the intracellular *A. actinomycetemcomitans* within 2 hours. In the same experimental conditions, treatment with amoxicillin at its peak therapeutic concentration (4 mg/mL) kills only 14% of the intracellular bacteria.

### 1.3 Patient evaluation for an antibiotic: overview

Although it is difficult to completely remove subgingival biofilm and root deposits with SRP, most patients with chronic periodontitis respond favorably to treatment with conventional SRP without antibiotics. However, some cases can derive an additional increment of clinical attachment gain or probing depth reduction from
combining systemic antibiotics with SRP. The literature provides guidance for predicting which patients could potentially benefit.

- Patients who exhibit a poor response to adequate scaling and root planing, with continuing loss of clinical attachment
- Patients who test positive for presence of *P. gingivalis* or *A. actinomycetemcomitans* in their subgingival biofilm
- Patients with severe chronic periodontitis and generalized deep pocket depths

There is agreement that patients who fail to respond favorably to SRP, especially those with progressive attachment loss, can benefit from treatment with antibiotics.\(^{24}\) As previously mentioned, progressive chronic periodontitis is often associated with persistent infections by *Porphyromonas gingivalis*, *A. actinomycetemcomitans*, and *Prevotella intermedia*,\(^{10}\) which invade the soft tissue wall of the periodontal pocket and are difficult to eliminate with SRP. Consistent with this recommendation, patients with chronic periodontitis who have undergone microbiological testing and are positive for *Porphyromonas gingivalis* or *A. actinomycetemcomitans* in their subgingival plaque can be expected to benefit from use of antibiotics.\(^{25}\) Finally, patients with generalized severe chronic periodontitis and multiple deep periodontal pockets may also benefit.\(^{26,27}\)

The common thread in these guidelines is an acknowledgment that SRP has limited ability to eliminate invasive pathogens and remove biofilm from inaccessible sites. Smokers typically exhibit a less favorable response to periodontal therapy than nonsmokers. There is evidence that subgingival pathogens are more difficult to eliminate in smokers.\(^{28,29}\) Although some studies have suggested that adjunctive systemic antibiotics can improve the responses to periodontal therapy in smokers,\(^{30}\) a recent systematic review concluded that additional well-designed randomized clinical trials are needed to provide sufficient evidence to support the use of adjunctive antibiotics in the treatment of periodontitis in smokers.\(^{31}\)

### 1.4 Efficacy of scaling and root planing as the sole treatment of Periodontitis

Although SRP is regarded as the gold standard of nonsurgical periodontal treatment, it is a highly demanding therapy. Its effectiveness is limited by anatomic
factors (furcation involvement, tooth type, and surface) and the experience of the operator.\textsuperscript{32} As previously mentioned, SRP loses some of its ability to eliminate subgingival biofilm as pocket probing depths increase.\textsuperscript{33,34} Despite this, the magnitude of probing depth reduction and clinical attachment gain resulting from SRP is greatest at periodontal sites with deep pretreatment probing depths (Table 3).\textsuperscript{35}

In pockets deeper than 6 mm, SRP provides a mean clinical attachment gain of 1.19 mm and a mean probing depth reduction of 2.19 mm. In pockets of moderate (4–6 mm) depth, the respective values are 0.55 mm and 1.29 mm. SRP also reduces clinical signs of inflammation. As an example, it reduces bleeding on probing to approximately 43\% of baseline levels.\textsuperscript{35} These outcomes can be consistently achieved with chronic periodontitis patients, independent of the types of instruments used (power-driven or manual).\textsuperscript{36,37} Patients with poor oral hygiene, smoking habits, or poor glycemic control exhibit a less favorable response to SRP.

\subsection*{1.5 Efficacy of systemic antibiotics as the sole treatment of periodontitis}

In patients with advanced chronic periodontitis, diligent treatment with SRP requires a substantial amount of time and effort. It may seem reasonable to consider using systemic antibiotics as a cost-effective alternative to SRP for eliminating subgingival bacteria. Although this question has been examined in several reviews,\textsuperscript{27,38,39} relatively few studies have been specifically designed to address it. As a monotherapy for chronic periodontitis, metronidazole can reduce probing depths, induce modest attachment gains, reduce bleeding on probing, and suppress spirochetes in subgingival biofilm.\textsuperscript{38} Comparisons of the efficacy of metronidazole alone with SRP have demonstrated that metronidazole is inferior or, at best, equivalent in improving periodontal status.\textsuperscript{40–42} Moreover, a meta-analysis of 4 clinical trials that compared attachment level changes in subjects with untreated periodontitis with that of subjects treated with metronidazole alone or metronidazole in combination with amoxicillin failed to show a statistically significant difference between groups. Thus, there is not sufficient evidence that systemic antibiotics, when used as a monotherapy, are beneficial in the treatment of periodontitis.\textsuperscript{27}
In contradistinction to these studies, a more recent study concluded that a combination of metronidazole and amoxicillin as the sole therapy for periodontitis produces changes in clinical and microbiological parameters that are similar to those obtained from conventional SRP.\textsuperscript{43} However, every subject in this study received supragingival scaling to facilitate periodontal probing. Thus, the group treated with antibiotics did not actually receive a monotherapy because removal of supragingival biofilm has been shown to alter the number and composition of subgingival bacteria.\textsuperscript{44}

Consistent with most clinical studies, microbiological studies have shown that bacteria living in biofilms are more resistant to antimicrobial agents than single, dispersed (planktonic) bacteria.\textsuperscript{45–47} This may be related to impairment of antibiotic diffusion into biofilms or to the slower bacterial growth rate secondary to deprivation of nutrients within the biofilm\textsuperscript{44}; however, there are other contributing factors. The close association of bacteria living in biofilms facilitates horizontal transfer of genetic information that confers resistance to antibiotics.\textsuperscript{48,49} In vitro studies have shown that the antibiotic concentrations found in gingival crevicular fluid (GCF) have limited impact on periodontal pathogens living in biofilms.\textsuperscript{50,51} For these reasons, there is a consensus that antibiotics should only be prescribed after biofilm is mechanically disrupted.

1.6 Efficacy of scaling and root planing combined with systemic antibiotics

Several comprehensive reviews have evaluated the efficacy of a combination of SRP and systemic antibiotics in treatment of chronic periodontitis.\textsuperscript{26,27,39,52–55} Their general conclusions are summarized below:

- Combining systemic antibiotics with SRP can provide a greater therapeutic benefit than SRP alone.
- The combination of antibiotics and SRP provides a greater benefit to patients with aggressive periodontitis than to those with chronic periodontitis.
- The combination of SRP and antibiotics yields its greatest benefit at sites with deep initial probing depths.
• Several different antibiotic regimens are capable of enhancing the treatment response to SRP. Meta-analyses support the use of metronidazole (alone or in combination with amoxicillin) or azithromycin.
• Indirect evidence suggests that antibiotics should be started on the day SRP is completed and that SRP should be completed within a short period of time (ideally, less than a week).

Meta-analysis is a useful statistical technique for combining results from different studies to achieve higher statistical power. This approach has been used to analyze the benefits of combining antibiotics with SRP. Table 4 summarizes data from several meta-analyses of the overall effect of combined treatment of chronic periodontitis with SRP and adjunctive antibiotics in comparison with treatment with SRP alone. These studies examined treatment effects throughout the mouth, including sites with only minor attachment loss and shallow probing depths. Based on 2 meta-analyses that considered the effects of a broad range of different antibiotic regimens on treatment of chronic periodontitis, combined therapy can enhance clinical attachment gain by 0.20 to 0.24 mm and decrease probing depth by a mean of 0.28 mm in comparison with SRP alone.\textsuperscript{27,54}

Neither of these analyses could identify an antimicrobial regimen that was clearly superior to the others. Adjunctive antibiotics seem to consistently enhance the clinical response to SRP for both aggressive and chronic periodontitis patients but patients with aggressive periodontitis seem to derive greater benefit. The mean clinical attachment gain observed in studies of subjects with aggressive periodontitis patients is nearly 3 times greater than that observed in studies of chronic periodontitis.\textsuperscript{27}

Regarding the effects of specific antibiotic regimens, treatment with SRP combined with amoxicillin and metronidazole can enhance overall clinical attachment gain by 0.16 to 0.21 mm, and overall probing depth reduction by 0.29 to 0.43 mm, in comparison with SRP alone (see Table 4).\textsuperscript{52,54} Similarly, the adjunctive benefits of combining metronidazole with SRP correspond to an additional 0.1 mm of attachment gain and 0.15 to 0.18 mm of probing depth reduction.\textsuperscript{54,55} The combination of SRP and azithromycin yields a mean attachment gain of 0.11 mm (not statistically significant) and a mean probing depth reduction of 0.39 mm in comparison with SRP alone.\textsuperscript{54} Meta-
analysis of studies using an adjunctive doxycycline regimen failed to demonstrate a significant overall enhancement of attachment gain or probing depth.\textsuperscript{54}

Evidence suggests that the benefits of combining antibiotics with SRP are more substantial at sites with initial probing depths of greater than 6 mm (Table 5). At deeper sites, treatment with SRP combined with amoxicillin and metronidazole can enhance clinical attachment gain by 0.45 to 0.67 mm and reduce mean probing depth by 0.92 mm in comparison with treatment with SRP alone.\textsuperscript{26,54} Combining metronidazole with SRP results in an additional attachment gain of 0.55 to 0.66 mm and an additional probing depth reduction of 0.83 mm in comparison with SRP alone.\textsuperscript{26,54} Use of azithromycin as an adjunct to SRP enhances mean attachment gain and probing depth reduction by 0.43 mm and 0.52 mm, respectively, over SRP alone.\textsuperscript{54}

Because many different protocols have been used in studies that evaluated the benefits of combining antibiotics with SRP, there is a lack of evidence pointing to a specific protocol. However, there is indirect evidence that antibiotic therapy should immediately follow the completion of SRP and that SRP should be completed within a reasonably short time (ideally, within 1 week).\textsuperscript{39}

1.7 Treatment complications and resistance

Systemic antibiotics have the potential to produce adverse reactions that must be considered in balance with their expected benefits (see Table 2). Direct toxic effects of amoxicillin, metronidazole, doxycycline, or azithromycin are rare. However, all have the potential to induce nausea, vomiting, diarrhea, and abdominal pain in a small percentage of patients.\textsuperscript{56} The most common adverse effects associated with amoxicillin and other penicillins are allergic reactions, including skin rashes; serum sickness; and, rarely, anaphylaxis.\textsuperscript{57} Patients taking metronidazole often report altered taste sensations and can experience Antabuse effects in response to alcohol ingestion.\textsuperscript{38} Photosensitivity can occur in individuals taking doxycycline.\textsuperscript{56} In rare instances, azithromycin may induce angioedema or cholestatic jaundice. In addition, azithromycin can contribute to cardiac arrhythmias and slightly increase the risk of cardiovascular death in individuals with a
high baseline risk of cardiovascular disease.\textsuperscript{58} Patients should be informed of the potential for adverse reactions; however, these effects typically present as gastrointestinal upsets and most are not serious.\textsuperscript{27,39}

Several fundamental issues can, individually or in combination, undermine the therapeutic benefits associated with use of adjunctive antibiotics in periodontal therapy. Lack of patient compliance (adherence) with the prescribed dosage regimen is a major concern. If the antibiotic does not reach optimal concentrations at the infection site or the duration of treatment is too short because the patient does not follow directions, its therapeutic benefit will be compromised. Studies have shown that compliance can be poor with complex regimens that require patients to take multiple doses per day.\textsuperscript{59} Thus, it is reasonable to expect that compliance with a combined regimen of amoxicillin and metronidazole will be lower than with a once-a-day azithromycin regimen.

Prescribing an antibiotic will not predictably enhance treatment outcomes if the subgingival biofilm is not thoroughly disrupted before antibiotic treatment or if patients fail to inhibit biofilm reformation by maintaining good oral hygiene.\textsuperscript{39,60} An antibiotic’s minimal inhibitory concentration (MIC) for bacteria living in a biofilm can be 10 to 1000-fold higher than for bacteria growing in a planktonic state,\textsuperscript{61,62} and typically exceeds the concentration that the antibiotic can attain in GCF. In effect, disruption of subgingival biofilm decreases the MIC values and renders the bacteria more susceptible to antibiotics at concentrations found in GCF.\textsuperscript{63} There is evidence to suggest that the red complex of subgingival bacteria associated with chronic periodontitis is relatively susceptible to antibiotics.\textsuperscript{27} However, other subgingival pathogens found in chronic periodontitis patients, including Prevotella intermedia, Prevotella nigrescens, and A. actinomycetemcomitans, are often resistant to doxycycline, amoxicillin, or metronidazole.\textsuperscript{64} Failure to eliminate these pathogens could limit the success of SRP combined with an adjunctive antibiotic regimen.

If examination reveals that inflammation has not resolved or attachment loss has not been arrested by treatment with a combined regimen of SRP and antibiotics within 2 to 3 months, 2 different approaches can be used to address resistance to treatment. Microbiological testing could help explain why the original treatment failed and guide
additional nonsurgical therapy. Subgingival plaque samples should be collected from progressive disease sites with sterile paper points and shipped to a laboratory that has the specialized expertise needed to identify pathogens that have not been eliminated. Based on this information, an alternative regimen that is appropriate for targeting the remaining pathogens can be selected. Because the subgingival environment progressively recolonizes with bacteria after SRP, subgingival biofilm must be disrupted and dispersed again before administering the alternative antibiotic. At the time the clinical response is reexamined 2 to 3 months later, it may be prudent to conduct another microbiological test to confirm that pathogens have been eliminated. As a second option, periodontal surgery may be used to address persistent pocketing and attachment loss.

There is general agreement that selective, rather than routine use of antibiotics is the best practice. Commensal bacteria living in the intestinal tract contribute to the development, maintenance, and function of the immune system. By disrupting commensal microbiota, antibiotics can perturb host defenses in a detrimental manner. Moreover, antibiotic resistance has become a serious public health issue in recent years. Its economic and social costs are significant. A recent study suggests that subgingival biofilm can serve as a reservoir of β-lactam resistance genes. Dentists can help prevent these issues by prescribing antibiotics only when they are indicated, by prescribing an appropriate antibiotic regimen, and by using antibiotics only after subgingival biofilm has been debrided. Patients can help reduce the risk of inducing resistance by complying with the recommended dosage and duration of the prescribed regimen.

**1.8 Evaluation of outcome and long-term recommendations**

Increased tooth survival is one of the most relevant outcomes for reporting the effectiveness of periodontal therapy, and one that patients readily understand. It is rarely used, however, because exceptionally long study periods are required to obtain meaningful data. As a practical matter, increases in clinical attachment level and reduction of probing depths are reasonable proxies for increased tooth survival. In absolute terms, a full-mouth attachment gain of only 0.10 to 0.24 mm (see Table 4) could be viewed as a modest benefit for using antibiotics in combination with SRP. Considering
that patients who are highly susceptible to periodontitis experience a mean full-mouth attachment loss of 0.067 mm per year during supportive therapy after active periodontal treatment, an attachment gain of 0.10 to 0.24 mm effectively offsets 1.5 to 3.5 years of disease progression.

At periodontal sites with deep (>6 mm) probing depths, combining an antibiotic with SRP can enhance attachment gain by 0.43 to 0.67 mm (see Table 5). Because SRP yields a mean attachment gain of 1.19 mm at sites with deep initial probing depths (see Table 3), the use of antibiotics in combination with SRP enhances attachment gain by 36% to 56% more than that obtained from SRP alone. In patients with many deep pockets, a benefit of this magnitude is cost-effective and clinically relevant because it could potentially decrease the need for periodontal surgical therapy.

As mentioned previously, patients with mild-to-moderate chronic periodontitis usually respond favorably to initial treatment with SRP alone without adjunctive antibiotics. Patients with severe chronic periodontitis who have multiple deep pockets, progressive attachment loss, or test positive for invasive subgingival pathogens may benefit from an initial therapy that combines systemic antibiotics with SRP. Consistent with the meta-analyses detailed in Tables 4 and 5, a combination of amoxicillin and metronidazole is a reasonable choice for adjunctive use in patients who are not allergic to b-lactam antibiotics. This combination of 2 bactericidal agents has the potential to inhibit a broader spectrum of bacteria than a single agent and is less likely to induce resistance. In patients who are allergic to amoxicillin, an adjunctive regimen of metronidazole or azithromycin is a reasonable alternative. The response to initial periodontal treatment should be evaluated within 2 to 3 months and adjusted as necessary. If progression of attachment loss has been arrested and the outcome is generally favorable, it would be appropriate to treat persistent deep pockets with periodontal surgery. Most patients treated with surgical therapy do not require a postoperative antibiotic regimen. Currently, there is not sufficient evidence to support the adjunctive use of systemic antibiotics in conjunction with periodontal surgery.

1.9 Summary
• Although chronic periodontitis often responds to mechanical debridement alone, patients who have progressive attachment loss, invasive subgingival pathogens, or deep pockets may benefit from combining systemic antibiotics with mechanical therapy.

• Bacteria in subgingival biofilm are resistant to antibiotics. Antibiotics should only be prescribed after biofilm has been mechanically disrupted, not as the sole approach to treatment. Meta-analyses suggest that metronidazole (alone or in combination with amoxicillin) or azithromycin produce statistically significant adjunctive benefits in combination with mechanical therapy.

• When used to treat chronic periodontitis, the combination of mechanical therapy and antibiotics yields its greatest benefit at sites with deep initial probing depths.

• Systemic antibiotics have the potential to produce adverse reactions that must be considered in balance with their expected benefits.

1.10 References


56. Walker CB. Selected antimicrobial agents: mechanisms of action, side effects and


Table 1. Characteristics of antibiotics used to treat chronic periodontitis

<table>
<thead>
<tr>
<th>Agent</th>
<th>Half-Life in Serum (h)</th>
<th>Action</th>
<th>GCF Level (µg/mL)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/mL) for <em>Porphyromonas gingivalis</em></th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/mL) for <em>T. forsythia</em></th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/mL) for <em>Prevotella intermedia</em></th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/mL) for <em>A. actinomycetemcomitans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>1–2</td>
<td>Bactericidal</td>
<td>3–4</td>
<td>&lt;0.016</td>
<td>0.38</td>
<td>0.25–1.5</td>
<td>0.4–1</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>6–12</td>
<td>Bactericidal</td>
<td>8–10</td>
<td>&lt;0.016</td>
<td>0.005</td>
<td>0.032–0.25</td>
<td>64–96</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>40–68</td>
<td>Bacteriostatic or bactericidal</td>
<td>3–10</td>
<td>0.094–0.5</td>
<td>0.5–1</td>
<td>0.25–0.4</td>
<td>0.875–4</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>6–8</td>
<td>Bacteriostatic</td>
<td>5–10</td>
<td>0.023–0.25</td>
<td>0.19</td>
<td>2–4</td>
<td>0.2–1.5</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>12–22</td>
<td>Bacteriostatic</td>
<td>2–8</td>
<td>0.047</td>
<td>0.38</td>
<td>0.05</td>
<td>1</td>
</tr>
</tbody>
</table>

Abbreviations: GCF, gingival crevicular fluid; MIC<sub>90</sub>, minimal inhibitory concentration of an antibiotic at which 90% of bacterial isolates are inhibited. Data from Refs. 11–1
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Prescription</th>
<th>Potential Adverse Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin + Metronidazole</td>
<td>500 mg tid for 8 d, 250 mg tid for 8 d</td>
<td>Hypersensitivity to amoxicillin, nausea, diarrhea, vomiting, altered taste sensations, Antabuse effect</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>500 mg tid for 7 d</td>
<td>Nausea, vomiting, altered taste sensations, Antabuse effect</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>500 mg qd for 3 d</td>
<td>Diarrhea, nausea, vomiting, abdominal pain, cholestatic jaundice, increased risk of serious cardiac arrhythmia (prolonged Q-T interval) Inhibition of bactericidal agents if used in combination</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>200 mg initial dose, then 100 mg qd for 21 d</td>
<td>Photosensitivity, nausea, diarrhea, vomiting, and abdominal pain Inhibition of bactericidal agents if used in combination</td>
</tr>
</tbody>
</table>

Table 2. Representative antibiotic regimens for adjunctive use in treatment of chronic periodontitis
Table 3. Summary of clinical outcomes achieved with scaling and root planing when used to treat nonmolar sites

Table 4. Meta-analyses of clinical outcomes associated with combining systemic antibiotics with scaling and root planing to treat chronic periodontitis (overall effects at all sites)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Dates and Number of Included Studies</th>
<th>Antibiotics Studied</th>
<th>Observation Time (mo)</th>
<th>Mean Clinical Attachment Level Gain (P Value)</th>
<th>Mean Probing Depth Reduction (P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haffajee et al,27 2003(^a)</td>
<td>1983–2001 n = 17</td>
<td>MET, SPIR, AMX + MET, AMX + CA, TET, DOX</td>
<td>&gt;1, most ~6</td>
<td>0.24 mm (0.001)</td>
<td>Not analyzed</td>
</tr>
<tr>
<td>Keestra et al,(^{54}) 2015(^b) (main analysis)</td>
<td>1994–2012 n = 35</td>
<td>AMX, AMX + CA, AMX + MET, MET, AZM, CLR, DOX, SDD, ORN, SPIR, TET, MOX</td>
<td>3</td>
<td>0.20 mm (0.0004)</td>
<td>0.28 mm (&lt;0.00001)</td>
</tr>
<tr>
<td>Sgolastra et al,(^{52}) 2012</td>
<td>2001–2011 n = 4</td>
<td>AMX + MET</td>
<td>≥3</td>
<td>0.21 mm (0.03)</td>
<td>0.43 mm (&lt;0.0001)</td>
</tr>
<tr>
<td>Keestra et al,(^{54}) 2015 (subanalysis)</td>
<td>1998–2012 n = 7</td>
<td>AMX + MET</td>
<td>3</td>
<td>0.16 mm (0.05)</td>
<td>0.29 mm (0.003)</td>
</tr>
<tr>
<td>Sgolastra et al,(^{55}) 2015</td>
<td>1998–2012 n = 6</td>
<td>MET</td>
<td>≥3</td>
<td>0.10 mm (&lt;0.00001)</td>
<td>0.18 mm (0.0001)</td>
</tr>
<tr>
<td>Keestra et al,(^{54}) 2015 (subanalysis)</td>
<td>2004–2012 n = 5</td>
<td>MET</td>
<td>3</td>
<td>0.10 mm (0.12)</td>
<td>0.15 mm (0.004)</td>
</tr>
<tr>
<td>Keestra et al,(^{54}) 2015 (subanalysis)</td>
<td>2005–2012 n = 6</td>
<td>AZM</td>
<td>3</td>
<td>0.11 mm (0.32)</td>
<td>0.39 mm (0.004)</td>
</tr>
<tr>
<td>Keestra et al,(^{54}) 2015 (subanalysis)</td>
<td>1999–2008 n = 4</td>
<td>DOX</td>
<td>3</td>
<td>0.09 mm (0.34)</td>
<td>0.11 mm (0.15)</td>
</tr>
</tbody>
</table>

Abbreviations: AMX, amoxicillin; AZM, azithromycin; CA, clavulanic acid; CLR, clarithromycin; DOX, doxycycline; MET, metronidazole; MOX, moxifloxacin; ORN, ornidazole; SDD, subantimicrobial-dose doxycycline; SPIR, spiramycin; TET, tetracycline. Data from Refs.\(^{26,52,54,55}\)

\(^a\) Main meta-analysis included 3 studies that examined the effect of the antibiotic as a sole treatment.

\(^b\) Main meta-analysis included 9 studies that examined the effect of adjunctive subantimicrobial-dose doxycycline.
Table 5. Meta-analyses of clinical outcomes associated with combining systemic antibiotics with scaling and root planing to treat chronic periodontitis (effects at sites with initial probing depths >6 mm)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Dates and Number of Included Studies</th>
<th>Antibiotics Studied</th>
<th>Observation Time (mo)</th>
<th>Mean Clinical Attachment Level Gain (P Value)</th>
<th>Mean Probing Depth Reduction (P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herrera et al,26 2002 (subanalysis)</td>
<td>1998 n = 2</td>
<td>AMX + MET</td>
<td>12–24</td>
<td>0.45 mm (0.001)</td>
<td>Not analyzed</td>
</tr>
<tr>
<td>Keestra et al,54 2015 (subanalysis)</td>
<td>2008–2012 n = 4</td>
<td>AMX + MET</td>
<td>3</td>
<td>0.67 mm (0.02)</td>
<td>0.92 mm (0.0003)</td>
</tr>
<tr>
<td>Herrera et al,26 2002 (subanalysis)</td>
<td>1983–1984 n = 2</td>
<td>MET</td>
<td>2–12</td>
<td>0.55 mm (0.057)</td>
<td>Not analyzed</td>
</tr>
<tr>
<td>Keestra et al,54 2015 (subanalysis)</td>
<td>2004–2012 n = 5</td>
<td>MET</td>
<td>3</td>
<td>0.66 mm (&lt;0.00001)</td>
<td>0.83 mm (&lt;0.00001)</td>
</tr>
<tr>
<td>Keestra et al,54 2015 (subanalysis)</td>
<td>2002–2012 n = 5</td>
<td>AZM</td>
<td>3</td>
<td>0.43 mm (0.03)</td>
<td>0.52 mm (0.0003)</td>
</tr>
</tbody>
</table>

Abbreviations: AMX, amoxicillin; AZM, azithromycin; MET, metronidazole.
Chapter 2

Azithromycin: overview

2.1 Introduction

Azithromycin is categorized as a macrolide antibiotic and is a semi-synthetic derivative of erythromycin. Through insertion of a methyl-substituted nitrogen in place of the carbonyl group at the 9a position of the aglycone ring, this 15-membered lactone ring azalide acquires improved pharmacological properties, including acid stability, lower incidence of adverse effects, longer half-life in serum, and greater tissue penetration.\(^1\),\(^2\) Compared to its predecessor, azithromycin has comparable antimicrobial activity against Gram-positive organisms and greater activity against Gram-negative bacteria.\(^3\) These properties together make azithromycin the most widely used broad-spectrum antimicrobial in North America.\(^4\) In addition, macrolide antibiotics exhibit immunomodulatory properties that are used in the treatment of disease distinct from bacterial infections, including diffuse panbronchiolitis, chronic obstructive pulmonary disease, cystic fibrosis, and non-cystic fibrosis bronchiectasis.\(^5\)-\(^8\) Since periodontitis is a condition in which bacterial infection and immuno-inflammatory responses are both critical factors, azithromycin may play a dual role in periodontal therapy by suppressing periodontal pathogens and modulating host inflammatory responses.

2.2 Antimicrobial properties

Similar to other macrolide antibiotics, azithromycin exerts its antimicrobial activity by binding to and interfering with 50S ribosomal subunit formation, and therefore inhibits synthesis of bacterial protein.\(^9\) Azithromycin also directly interacts with the outer membranes of bacteria by displacement of divalent cations (Ca\(^{2+}\) and Mg\(^{2+}\))
from their binding sites on lipopolysaccharides, thereby destabilizing the outer membranes and increasing its permeability.\textsuperscript{10}

The antimicrobial functions of azithromycin are multifaceted. In periodontal diseases and other human diseases, bacteria have been shown to form a biofilm where a diverse microbial community is embedded in a matrix of host and bacterial extracellular polymeric substances.\textsuperscript{11} Bacteria living in biofilms acquire resistance to antimicrobial agents through several mechanisms and become a critical issue in antimicrobial chemotherapy. Azithromycin can inhibit the formation of bacterial biofilm at sub-MIC concentrations (lower than the minimum concentrations required to inhibit bacterial growth),\textsuperscript{12, 13} and this could be in part due to reduction of the expression of the gene products necessary for biofilm formation.\textsuperscript{14} This could also result from reduction of bacterial adhesion, the first step for bacterial colonization.\textsuperscript{15} In established biofilms, azithromycin appears to penetrate deeply and kill bacteria, although the complete destruction of biofilms is not observed.\textsuperscript{12}

Quorum sensing is an inter-bacterial communication process by which signal molecules act as autoinducers to initiate a series of biological functions when a certain population density is reached.\textsuperscript{16} It helps to coordinate gene expression among members in the community and enhances virulence of these pathogens in both animals and humans.\textsuperscript{17} Studies indicate that azithromycin not only antagonizes quorum-sensing, but also inhibits alginate polymer production by \textit{Pseudomonas aeruginosa}.\textsuperscript{16, 18} In addition, treatment with sub-MIC concentrations of azithromycin results in loss of bacterial mobility provided by flagella and fimbriae, and this reduction facilitates phagocytosis and killing by macrophage.\textsuperscript{19}

\textbf{2.3 Pharmacokinetics}

Azithromycin has relatively low bioavailability (17\% to 37\%) after oral administration, mainly due to incomplete absorption rather than acid degradation or extensive first-pass metabolism.\textsuperscript{20, 21} In contrast to the instability of erythromycin under the acidic conditions of the stomach,\textsuperscript{22} azithromycin possesses an extra nitrogen atom that prevents rapid degradation by blocking the internal dehydration pathway, leading to a 300-fold increase in stability over the gastric pH range.\textsuperscript{23} Azithromycin reaches its peak
serum concentrations of 0.35-0.45 µg/ml 2 hours after a single oral 500 mg dose. The relatively low serum concentrations appear to be the results of a quick blood-tissue distribution (with a large volume of distribution of 25 to 35 l/kg). The major route of elimination is biliary excretion and trans-intestinal secretion although about 6 – 12 % of azithromycin is found unchanged in the urine. Unlike other macrolides, azithromycin is not metabolized by cytochrome P450 enzymes and does not affect the pharmacokinetics of drugs that are substrates of cytochrome P450 enzymes. The terminal serum half-life of azithromycin ranges from 40 to 78 hours. The prolonged serum half-life could be partly explained by the slow efflux of drug from body tissues back into serum, including prostate, tonsil, gingiva and bone.

A high degree of cellular accumulation of azithromycin has been reported in various mammalian cells, including epithelial cells, fibroblasts, and phagocytes. The intracellular concentrations in human neutrophils could be more than 200-fold higher than the extracellular concentrations. The active uptake by phagocytes facilitates the delivery of drug from circulation to sites of inflammation. In addition, due to their wide distribution in human body, fibroblasts could serve as potential reservoirs for azithromycin and maintain high local concentrations. The exact mechanism of cellular accumulation is not fully understood. Some people believe that passive trans-membrane transport followed by protonation and trapping of azithromycin in acidic organelles is a potential mechanism because neutralization of lysosomal pH markedly increases antibiotic efflux. The dependence of accumulation on temperature, cell viability, pH, and its reduction by 2,4-dinitrophenol (a metabolic inhibitor) suggest the existence of an active transport system. Besides, azithromycin is confirmed to be a substrate of P-glycoprotein and compete with other substrates of this transporter protein, including verapamil and cyclosporine A. It is possible that both active and passive mechanisms are involved.

2.4 Immuno-modulatory properties

Although the non-antimicrobial effects of macrolide antibiotics were suspected a long time ago, these properties were not extensively addressed until the recent 3
The efficacy of erythromycin on chronic inflammatory pulmonary diseases was found to be independent of its anti-bacterial effect. Azithromycin, similar to many of its kind, has shown promising immuno-modulatory effects on various chronic inflammatory conditions through interaction with structural cells as well as leukocytes.

Azithromycin increases the transepithelial electrical resistance on human airway epithelia by changing the processing of tight junction proteins claudin-1 and claudin-4 in a dose-dependent manner, and this could reduce *Pseudomonas aerugiosa* binding and prevent abnormal transepithelial ion transport. Mucus hypersecretion, a common feature of many chronic airway diseases, is also inhibited by azithromycin by interfering with intracellular signal transduction. Cytokines are a broad category of hormone-like small proteins that play an important role in inter-cellular communication and cellular activities, especially in the immune system. They induce changes in not only nearby cells (paracrine signaling) but also the same cells where they originate from (autocrine signaling). Pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, tumor necrotic factor (TNF)-α, and interferon-γ and chemokines (cytokines with a strong chemotactic capacity) such as IL-8 boost the immuno-inflammatory responses through positive feedback loops. Anti-inflammatory cytokines including IL-10 and transforming growth factor (TGF)-β lessen the responses through negative feedback loops. A variety of cell types are involved with production of cytokines, including neutrophils, macrophages, lymphocytes, epithelial cells and fibroblasts. The effect of azithromycin on cytokines has been extensively reviewed. The majority of studies suggest that azithromycin, along with other macrolides, down-regulates the production of pro-inflammatory cytokines and promotes the secretion of anti-inflammatory cytokines.

Besides the effects on cytokine production, azithromycin modulates host immune responses in other ways. Azithromycin alters macrophage phenotype, leading to a shift from classically activated macrophages (M1 cells that actively secrete pro-inflammatory cytokines and kill invading microorganisms) toward alternatively activated macrophages (M2 cells that direct a Th2 humoral responses and coordinate repair following inflammation). It also enhances phagocytosis of apoptotic bronchial epithelial cells and neutrophils, possibly through increased expression of mannose receptor CD206 on
alveolar macrophages.\textsuperscript{46, 47} In neutrophils, the oxidative burst capacity (the production and release of reactive oxygen species to enhance the cytotoxic capability) is profoundly inhibited by azithromycin.\textsuperscript{48} The reduced neutrophil numbers in bronchoalveolar lavage fluid after azithromycin administration could also be a result of reduced reaction to chemokines, which is found in another closely-related macrolide, clarithromycin.\textsuperscript{49, 50} The pro-apoptotic effect of azithromycin on neutrophils and activated lymphocytes has been associated with reduced levels of tissue inflammation.\textsuperscript{51, 52}

Studies on human epithelium and phagocytes indicate that azithromycin may modulate immune responses through transcription factors NF-κB and AP-1.\textsuperscript{53-55} The NF-κB transcription factor exists as heterogeneous dimers composed of proteins of the NF-κB/Rel family. It has been shown to regulate the expression of proinflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules, and is recognized as a key mediator of human immune responses.\textsuperscript{56} The AP-1 transcription factor is also a dimeric complex consisting of members of the Jun, Fos, ATF (activating transcription factor) and MAF (musculoaponeurotic fibrosarcoma) protein families. It plays an important role in cell proliferation, differentiation and cell transformation during development as well as in adult tissues, and may regulate inflammatory responses through expression of cytokines.\textsuperscript{57, 58} Other studies indicate the modulation may be secondary to direct effects of azithromycin on upstream signaling processes such as Erk1/2 MAP kinase pathway and on a signaling GTPase, Ras-related C3 botulinum toxin substrate 1 (Rac-1).\textsuperscript{42, 54, 59}

### 2.5 Application in the field of medicine

Due to its favorable tissue distribution and broad antimicrobial spectrum that covers many respiratory pathogens, azithromycin has been used effectively in the treatment of upper and lower respiratory tract infection.\textsuperscript{60, 61} Azithromycin, along with other macrolide antibiotics, may exert their benefits in viral infection through their immuno-modulatory properties.\textsuperscript{62} For example, azithromycin may enhance anti-viral defense by significantly increasing rhinovirus 1B- and 16-induced interferons and interferon-stimulated gene expression in human bronchial epithelial cells.\textsuperscript{63} As mentioned
earlier, the immuno-modulation from azithromycin has been largely used to treat diffuse panbronchiolitis, chronic obstructive pulmonary disease, cystic fibrosis and non-cystic fibrosis bronchiectasis, and post-transplant bronchiolitis.\textsuperscript{64}

Chronic bacterial prostatitis is hard to treat because few antimicrobial agents could distribute to the prostatic tissue and reach therapeutic concentrations at the site of infection. While fluoroquinolones are current drugs of choice, azithromycin has shown its efficacy in treatment of chronic bacterial prostatitis, especially those associated with obligate intracellular pathogens.\textsuperscript{65} Azithromycin is used to treat a number of sexually transmitted diseases. In the CDC’s 2010 treatment guideline, ceftriaxone plus azithromycin was recommended as drugs of choice for gonorrhea.\textsuperscript{66} A single dose of 1 gram azithromycin is the first-line drug for \textit{Chlamydia trachomatis} infection. Azithromycin as a 1.5\% ophthalmic solution in the treatment of bacterial or trachomatous conjunctivitis has also been reviewed.\textsuperscript{67}

\section*{2.6 Application in field of dentistry}

Although not commonly used, azithromycin has drawn the attention of dentists over the last 15 years due its favorable pharmacological properties. Its efficacy as an adjunctive antibiotic to non-surgical periodontal treatment (scaling and root planing) has been reviewed recently.\textsuperscript{68, 69} These reviews indicate that adjunctive azithromycin significantly improves reduction of probing depth, clinical attachment level, and bleeding on probing, and reduces the loadings of periodontal pathogens in patients with chronic or aggressive periodontitis. In addition, azithromycin has been associated with resolution of cyclosporin A-induced gingival overgrowth. The exact mechanism by which azithromycin reduces gingival overgrowth is still unclear. In rat gingiva, the overgrowth was associated with decreased collagen degradation due to reduced phagocytic activity of fibroblasts by cyclosporine A, while azithromycin partially compensated for this effect.\textsuperscript{70} Another study using human gingival fibroblasts indicated that azithromycin may improve gingival overgrowth by blocking cyclosporine A-induced fibroblast proliferation and collagen production and by recovering the reduced matrix metalloproteinase-2 activity.\textsuperscript{71}
Another common application of azithromycin in dentistry is antibiotic prophylaxis. According to the 2008 American Heart Association guideline on prevention of infective endocarditis, susceptible individuals should take either clindamycin 600 mg or azithromycin 500 mg 1 hour prior to procedures if allergic to penicillin. An azithromycin regimen started 3 days prior to scaling and root planing shows significant reduction of the incidence of bacteremia. In a recent randomized clinical trial, a single prophylactic dose of azithromycin prior to dental implant placement enhanced resolution post-operative inflammation to a greater extent than amoxicillin. This could potentially contribute to a faster post-surgical healing with a lower incidence of complication.

### 2.7 Adverse effects

A general concern of all antimicrobial agents is antibiotic resistance. Along with the increase clinical use, the resistance to azithromycin has increased in recent years especially in Japan and some European countries. Resistance of commensal *Staphylococcus aureus* to azithromycin is only secondary to that to penicillin, ranging from 1.6% in Sweden to 16.9% in France. Long-term azithromycin therapy in treatment of chronic lung disease increases the risk of bacterial resistance by 2.7 fold while decreasing bacterial colonization by approximately 45%. Even a 3-day course in periodontal treatment significantly increases the percentage of resistant isolates for up to 6 months.

The most common adverse effects of azithromycin are associated with gastrointestinal disturbance, with an average rate of 9.6%. Most of the gastrointestinal complaints are mild to moderate and do not require discontinuation of treatment. Some other rare adverse effects include rash (<1%) and hepatotoxicity related to a transient increase in liver enzymes. Other noteworthy, though rare, adverse effects include ototoxicity and cardiac toxicity. Meta-analysis indicates that patients receiving long-term azithromycin therapy are at risk of increased hearing impartment (risk ratio: 1.17, [95% CI, 1.030, 1.325]), although the impairment resolved in some patients after they continued to take azithromycin.
In an electrocardiogram, the QT interval corresponds to depolarization and repolarization of the ventricles. A prolonged QT interval usually indicates delayed ventricular repolarization whose primary drive is the rectified potassium current that regulates outward flow of potassium from the myocytes.\textsuperscript{81} While most macrolides pose a risk of QT prolongation by directly inhibition of the rectified potassium current and interference with cytochrome p450 metabolism of other QT-prolonging drugs,\textsuperscript{82} azithromycin has the least current inhibition and does not interact with cytochrome p450 among macrolides. The concern for cardiotoxicity associated with azithromycin has increased tremendously after the publication of a pivotal observational study on Tennessee Medicaid patients.\textsuperscript{83} The authors reported a small absolute increase in cardiovascular deaths after 5 days of azithromycin therapy compared to no antibiotics or amoxicillin, especially among patients with a high baseline risk of cardiovascular disease. However, the lack of information regarding concurrent medication use, comorbidities, and indication for antibiotic prescription somewhat limits the generalizability of the outcome. Svanstrom and colleagues did not find an increased risk of cardiovascular death associated with azithromycin in a Danish general population of young and middle-aged adults, although in their study azithromycin and penicillin V both showed an increased risk of death from cardiovascular causes compared to no use of antibiotics.\textsuperscript{84} While the fine balance between risk and benefits always needs to be prudently weighed, it has been suggested that administration of azithromycin in patients with high baseline risks of cardiovascular disease and co-administration of other known QT-prolonging medications should be avoided.\textsuperscript{85}

2.8 Outline of studies

In this project, we aim to characterize azithromycin transport by human cells native to periodontium and evaluate the effects of transportation on tissue distribution and invasive periodontal pathogens. In Chapter 3, a pharmacokinetic study was conducted. Individuals with clinically healthy periodontium received systemic administration of azithromycin and its concentrations in gingival crevicular fluid and serum were measured by agar diffusion bioassays.
In Chapters 4, 5, and 6, respectively, separate in vitro studies using human oral epithelial cells, neutrophils, and gingival fibroblasts were conducted, respectively. These three studies were conducted with a similar approach. Azithromycin transport by these cell types was characterized first, and in vitro infection studies by either Aggregatibacter actinomycetemcomitans or Porphyromonas gingivalis were conducted to evaluate the effect of intracellular accumulation on invasive pathogens.

In Chapter 7, the concluding remarks of this project and future research directions are presented.

2.9 References


Chapter 3

Azithromycin concentrations in blood and gingival crevicular fluid after systemic administration

3.1 Abstract

Background: Azithromycin is a macrolide antibiotic that is active against several periodontal pathogens. Macrolides are taken up and concentrated inside gingival fibroblasts, which could influence their pharmacokinetics. This study tested the hypothesis that steady-state levels of azithromycin are higher and more sustained in gingival crevicular fluid (GCF) than in serum.

Methods: Four healthy subjects received an initial dose of 500 mg azithromycin followed by 250 mg doses on each of the next 2 days. Serum and GCF samples were obtained 2 hours after the last dose (day 2) and on days 4 and 7. GCF samples were collected from maxillary posterior sites with paper strips. The strips were pooled and eluted with high purity water. After extraction, the azithromycin content of the serum samples and GCF eluates was determined with an agar diffusion bioassay.

Results: On days 2, 4 and 7, the concentrations of azithromycin in blood serum were 0.22 ± 0.02, 0.08 ± 0.02 and 0.04 ± 0.01 µg/ml, respectively. The concentrations in GCF were 8.82 ± 1.25, 7.90 ± 1.72 and 7.38 ± 1.15 µg/ml, respectively. Mean GCF levels were significantly higher than mean serum levels (P ≤ 0.02, paired t-test).

Conclusion: The findings demonstrate that the pharmacokinetic profiles of azithromycin are different in GCF and serum. At steady state, azithromycin concentrations in GCF were higher and more sustained than those in serum. Based on previous studies, the


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levels observed in GCF were above the MIC for *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, and Prevotella intermedia*.

### 3.2 Introduction

Elimination of bacterial plaque from root surfaces is a major goal of periodontal therapy. Although it usually halts progression of attachment loss, this therapeutic objective can be challenging in patients who are infected by invasive subgingival bacteria. *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, and Prevotella intermedia*, which can invade pocket epithelium,\(^1\)-\(^4\) are particularly difficult to eliminate by scaling and root planing. Use of adjunctive antibiotics is a logical approach for eradicating these pathogens and enhancing the response to scaling and root planing.\(^5\) Azithromycin is a macrolide antibiotic that inhibits a variety of subgingival bacteria.\(^6\)-\(^9\) Clarithromycin, a closely related macrolide, is actively transported and concentrated inside oral epithelial cells and gingival fibroblasts.\(^10\) Antibiotic uptake by host cells could provide several benefits in the treatment of periodontitis. Elevated macrolide concentrations inside oral epithelial cells could facilitate the killing of invasive pathogens. Since gingival fibroblasts are a relatively large cellular compartment of the gingiva,\(^11\) macrolide accumulation by these cells could allow them to function as drug reservoirs that enhance and sustain therapeutic concentrations at that site. Previous studies have shown that azithromycin and clarithromycin can attain higher steady-state levels in gingival tissue than in serum and suggest that tissue levels are increased in the presence of inflammation.\(^12, \, 13\) Azithromycin concentrations in gingiva reportedly persist for up to 14 days after systemic administration.\(^14\)

Gingival crevicular fluid (GCF) originates from the vessels of the gingival plexus, within the gingival connective tissue. It seeps through gingival connective tissue and passes through the junctional epithelium prior to entering the gingival crevice.\(^11\) Following systemic administration, we hypothesize that steady-state azithromycin concentrations in GCF are higher and more sustained than the corresponding concentrations in blood. The present study tested this hypothesis, using methods adapted to work with small samples of GCF.
3.3 Material and methods

Patient Population

Four healthy adult volunteers (three males and one female, mean age: 30 years) with no clinical periodontal attachment loss (AL) were recruited from the student population of The Ohio State University College of Dentistry. Written informed consent was obtained before participation. The study protocol and participant recruitment procedures were reviewed and approved by the Institutional Review Board at The Ohio State University, Columbus, Ohio.

Study Design

One week before administration of azithromycin, all participants had their teeth cleaned and received oral hygiene instruction. To obtain steady-state levels of azithromycin in periodontal tissues, participants were given an initial dose of 500 mg with subsequent 250-mg doses at 24 and 48 hours. Using an established protocol, GCF samples were collected and pooled from 12 maxillary posterior interproximal sites (the mesio-facial and mesio-lingual aspects of all maxillary first and second premolars and first molars) immediately before the first dose of azithromycin (day 0), 2 hours after the last dose on day 2, and on days 4 and 7 after the initial dose. These sites were selected because they were readily accessible and easy to protect from contamination by saliva. Before GCF collection, the collection sites were isolated with cotton rolls; supragingival plaque was removed (if present); and the site was gently air-dried. GCF was collected with paper strips (PerioPaper, Oraflow, Smithtown, NY) positioned at the orifice of the crevice for 30 seconds. GCF volumes were determined with a calibrated gingival fluid measurement device (Periotron 6000, IDE Interstate, Amityville, NY) and pooled. The gingival index and plaque index were assessed at the collection sites at every study visit. In addition, blood samples were obtained by venipuncture on days 2, 4, and 7. Blood serum and pooled GCF samples were stored at -20 °C in sealed vials.
Sample Analyses

Before analyses, GCF was eluted from the pools of paper strips with 200 mL of ultrapure water, using a method previously described. The efficiency of elution, as assessed with [3 H]-labeled macrolide (American Radiolabeled Chemicals, St. Louis, MO), was 67.4% ± 2.1% (data not shown). GCF eluates and blood serum samples (200 mL) were treated with 40 mL of 0.5 g/mL Na₂CO₃ and extracted three times with 1 mL diethyl ether. The extracts were dried, reconstituted in acetonitrile, and applied to sterile paper disks (BD Biosciences, Sparks, MD). After complete evaporation of the acetonitrile, the azithromycin content of the disks was determined with an agar diffusion bioassay, using Kocuria rhizophila (ATCC 9341, American Type Culture Collection, Manassas, VA) as the indicator organism. The assay was calibrated with 2 to 18 ng of authentic azithromycin (US Pharmacopeia, Rockville, MD). For GCF samples, calculations for azithromycin content incorporated a correction for the observed elution efficiency.

Statistical Analyses

The paired t test was used to evaluate differences in azithromycin concentration in GCF and blood. Based on a projected difference of 4 m g/mL in the mean azithromycin concentrations in GCF and blood serum and a pooled standard deviation of 1.5, the estimated number of participants required to achieve a power of 0.80 with an α of 0.05 in a paired t test was four. Repeated measures analysis of variance was used to examine the statistical significance in changes in azithromycin concentration, GCF azithromycin content, and GCF volume over the course of the study. The Holm-Sidak test was used for post hoc comparisons. In all statistical analyses, the statistical unit was the participant rather than the site.

3.4 Results

Consistent with maintenance of gingival health, the median PI and GI values were 0 throughout the course of the study (Table 6). After administration of azithromycin, there was a persistent and statistically significant reduction in the pooled volume of GCF
collected from the study sites (P < 0.001, repeated measures ANOVA, power of test = 0.99, Figure 1A). The pooled volumes on days 2, 4 and 7 were significantly lower than the baseline level (P < 0.05, Holm-Sidak test). On day 2, the azithromycin concentrations in GCF and blood serum were 8.82 ± 1.25 and 0.22 ± 0.02 µg/ml, respectively. Over the next five days, these concentrations decreased to 7.38 ± 1.15 and 0.04 ± 0.01 µg/ml, respectively (Figure 1B). Although concentrations in GCF did not decrease significantly between day 2 and day 7, there was a significant decrease in serum concentrations on days 4 and 7 (P < 0.05, Holm-Sidak test). Mean GCF levels were significantly higher than mean serum levels on days 2, 4 and 7 (P ≤ 0.02, paired t-test, power of tests ≥ 0.84). The rate of azithromycin infusion into the gingival crevice, as assessed by the amount recovered per 30 second pooled GCF sample, did not change significantly between days 2 and 7 (Table 6).

3.5 Discussion

The findings support the hypothesis that steady-state azithromycin concentrations in GCF are significantly higher and more sustained than those in serum. Azithromycin concentrations in GCF were more than 40-fold higher throughout the course of the study, and they decreased at a slower rate than the levels in serum. Azithromycin levels in GCF decreased by approximately 20% between the second and seventh days, while the levels in serum decreased by 80% during the same period. This may be attributed to the low degree of azithromycin binding to plasma proteins in combination with active accumulation of azithromycin by cells in peripheral tissues.18, 19 Gingival connective tissue contains a large volume of fibroblasts, which could serve as reservoirs for maintaining high local levels of azithromycin. Oral epithelial cells and polymorphonuclear leukocytes may also accumulate azithromycin at this site.10, 20 Our findings are consistent with a previous report that azithromycin concentrations in gingival tissue are up to 25-fold higher than the corresponding concentrations in blood.21 Macrolides are not the only antimicrobial agents that have a propensity to concentrate in GCF. Tetracyclines (e.g., doxycycline) and fluoroquinolones (e.g., ciprofloxacin) reportedly attain GCF concentrations that are several-fold higher than their
concentrations in serum.\textsuperscript{15, 22-24} The pharmacological properties of all these agents are presumably influenced by their ability to be taken up, sequestered and released by fibroblasts, leukocytes, and other types of cells.\textsuperscript{10, 20}

Azithromycin concentrations observed in GCF are substantially higher than the minimal inhibitory concentration (MIC) previously reported for several periodontal pathogens, including \textit{Aggregatibacter actinomycetemcomitans} (0.25-2.0 µg/ml),\textsuperscript{6} \textit{Porphyromonas gingivalis} (0.125-1 µg/ml),\textsuperscript{7, 8} \textit{Prevotella intermedia} (0.03-1 µg/ml), and \textit{Peptostreptococcus micros} (0.5-1 µg/ml).\textsuperscript{8}

Moreover, azithromycin concentrations remained above the MICs for these pathogens over the entire five-day observation period. While many antibiotics can be classified as having either a concentration dependent killing effect or a time dependent killing effect, bacterial killing by azithromycin is not solely dependent on either model (add Jain and Danziger ref).\textsuperscript{25} The time that target organisms are exposed to azithromycin concentrations above the MIC appears to be the best index of efficacy.\textsuperscript{26} Azithromycin also exhibits a prolonged post-antibiotic effect on inhibition of bacterial regrowth.\textsuperscript{26} Thus, pathogens found within periodontal tissues or in periodontal pockets sites that have been treated to disrupt subgingival biofilm should be vulnerable to inhibition by azithromycin. Pathogens living in native subgingival biofilm could be more difficult to inhibit at the azithromycin concentrations observed in this study. However, studies with an in vitro periodontal biofilm model suggest that azithromycin can penetrate the biofilm surface and partially dissolve the biofilm.\textsuperscript{27} Azithromycin also appears to dissolve biofilm associated with diffuse panbronchiolitis.\textsuperscript{28}

A limited number of randomized, placebo-controlled clinical trials have suggested that azithromycin is a useful adjunct to scaling and root planing (SRP) in the treatment of periodontitis. In a study of patients with aggressive periodontitis, the combination of SRP plus azithromycin resulted in a higher percentage of teeth with attachment gain ≥ 1 mm and a greater reduction in probing depths than SRP alone.\textsuperscript{29} In patients with chronic periodontitis, the combination of SRP plus azithromycin yielded a significantly greater reduction of probing depths for pockets initially 4 mm or more than SRP alone.\textsuperscript{30} A later study of non-surgical treatment of chronic periodontitis in smokers also demonstrated
that adjunctive use of azithromycin with SRP resulted in enhanced pocket depth reduction and clinical attachment gain at moderate (4 to 6 mm) and deep (>6 mm) periodontal sites when compared to SRP alone. Moreover, a recent study of subjects with *P. gingivalis*-associated chronic periodontitis demonstrated that, when compared with SRP alone, SRP combined with adjunctive azithromycin yielded significantly enhanced pocket depth reduction and attachment gain and a significant decrease in the detection of *P. gingivalis*.

Due to their anti-inflammatory activity, macrolides have been used in immunomodulatory therapy for chronic inflammatory lung diseases. Azithromycin also appears to produce anti-inflammatory effects in gingiva, as evidenced by its ability to reduce GCF volume and the GCF content of pro-inflammatory cytokines IL-1β, IL-8 and TNF-α. The mechanism for these effects appears to involve modulation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). Since GCF volume is strongly correlated with histological signs of gingival inflammation, the significant reduction of GCF volume observed between days 2 and 7 in this study is consistent with the previous report. The apparent anti-inflammatory effects of azithromycin could represent an additional benefit when applied to treatment of inflammatory periodontal diseases. In conclusion, the results demonstrate that systemic administration of azithromycin produces relatively high and sustained levels in GCF and provide a rationale for further clinical evaluation of its adjunctive benefits in the treatment of periodontitis.

### 3.6 References


Table 6. Clinical and pharmacologic observations during the study

Within each column, there were no statistically significant differences between the observed values.
* Median and range observed in 4 subjects
† Mean ± SEM.
Figure 1. Changes in GCF volume and azithromycin concentrations observed during the study

Vertical arrows indicate times when azithromycin was administered. A: Gingival crevicular fluid volumes collected during the study. The data represent the mean (± SEM) pooled GCF volume collected for 30 seconds from 12 maxillary premolar and first molar sites. B: Azithromycin concentrations in GCF and blood serum. The mean concentrations observed in GCF were significantly higher than those in serum on days 2, 4 and 7 (P ≤ 0.02, paired t-test)
Azithromycin kills invasive *Aggregatibacter actinomycetemcomitans* in gingival epithelial cells

4.1 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 intraepithelial *A. actinomycetemcomitans*. $[^{3}]$H-azithromycin transport by Smulow-Glickman gingival epithelial cells and SCC-25 oral epithelial cells was characterized. To test our hypothesis, we infected cultured Smulow-Glickman cell monolayers with *A. actinomycetemcomitans* (Y4 or SUNY 465 strain) for 2 h, treated them with gentamicin to eliminate extracellular bacteria, and then incubated them with azithromycin for 1 to 4 h. 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 $\mu$g/ml azithromycin yielded steady-state intracellular concentrations of 144 $\mu$g/ml in SCC-25 cells and 118 $\mu$g/ml in Smulow-Glickman cells. Azithromycin induced dose- and time-dependent intraepithelial killing of both *A. actinomycetemcomitans* strains. Treatment of infected Smulow-Glickman cells with 0.125 $\mu$g/ml azithromycin killed approximately 29% of the intraepithelial CFU of both strains within 4 h, while treatment with 8 $\mu$g/ml azithromycin killed $>82$% of the
CFU of both strains (P<0.05). Addition of carnitine inhibited the killing of intracellular bacteria by azithromycin (P<0.05). Thus, human gingival epithelial cells actively accumulate azithromycin through a transport system that facilitates the killing of intraepithelial *A. actinomycetemcomitans* and is shared with organic cations.

### 4.2 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 the 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 recolonize 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 extracrevicular reservoirs of *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 the elimination of pathogens from subgingival and extracrevicular 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 proinflammatory cytokines in gingival crevicular fluid (GCF) (11). Several clinical trials have demonstrated promising clinical and microbiological benefits of AZM in the treatment of 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 that 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 the elimination of two different strains of A. actinomycetemcomitans from cultured gingival epithelium.

4.3 Material 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 cells were grown in 50% DMEM–50% Ham’s F-12 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 (BHI) 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 salt 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 a concentration of 10 µg/ml for time course assays and at 10 to 50 µg/ml for kinetic assays to determine the Michaelis constant (Km) and maximal velocity of transport (Vmax). After the indicated interval (1 to 20 min for uptake time course assays 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-dibutyl phthalate (3:10), and centrifuged for 30 s 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 (1 µCi/ml; PerkinElmer, Waltham, MA) (23).

Several organic cations and organic acids were examined for the potential to inhibit AZM transport. All were purchased from Sigma Chemical Company (St. Louis, MO). Individual agents were added to 0.5-ml cell suspension aliquots simultaneously with [³H]-AZM. Lineweaver-Burk analysis was used to determine the mechanisms of inhibition.
Effect of AZM on killing of intraepithelial *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 lyses the vacuolar membrane soon after entry (25). Microtubules play a critical role in cell invasion by *A. actinomycetemcomitans*. Paclitaxel (originally named taxol), which stabilizes polymerized microtubules, enhances invasion and inhibits the subsequent exit of *A. actinomycetemcomitans* from infected epithelial cells. Thus, the assay was conducted with DMEM containing 10 µM paclitaxel (Sigma Chemical Company, St. Louis, MO) to help maintain the intracellular *A. actinomycetemcomitans* levels 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 1,000. After infection for 2 h 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 h. After the removal of gentamicin-containing medium, the infected monolayers were washed five 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 h. As a positive control, infected monolayers were cultured under identical condition in the absence of AZM. At the indicated intervals, monolayers were washed four times with HBSS and lysed in sterile water to release intracellular *A. actinomycetemcomitans*. Dilutions of the lysate were plated on BHI agar for the enumeration of surviving CFU. 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*. 
4.4 Results

Epithelial AZM transport

AZM accumulation by SCC-25 and SG cells was saturated within 20 min (Fig. 2), resulting in steady-state intracellular concentrations that were more than 20-fold higher than the extracellular concentrations (Table 7). Transport activity exhibited Michaelis-Menten kinetics (Fig. 2, inset). The observed Km 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 7). At steady state, with an extracellular AZM concentration of 5 µg/ml, the cellular/extracellular concentration ratio of SCC-25 cells was slightly higher than that of 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 8 and Fig. 2 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, bromosulfophthalein, hydrocortisone, taurocholate, and estrone-3-sulfate produced little or no inhibition of AZM transport. Treatment with 10 µM paclitaxel, used to enhance epithelial infection by A. actinomycetemcomitans, also had no significant effect on AZM transport by SG cells.

Killing of intraepithelial 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 GCF (2 to 8 µg/ml) (27). AZM produced dose- and time-dependent intraepithelial killing of both strains (Fig. 3, P < 0.001, repeated-measures analysis of variance). Cells infected with SUNY 465 required 4 h 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 h, while treatment with ≥2 µg/ml produced significant killing after 1 h (P < 0.01). Treatment with 8 µg/ml AZM killed approximately 49% of the control SUNY 465 CFU after 1 h, 83% after 2 h, and 85% after 4 h (P < 0.01, Holm-Sidak test). Under similar experimental conditions, treatment for 2 h with 4 µg/ml AMX killed only 14% of the SUNY 465 CFU (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 (Fig. 3, lower panel). Interestingly, treatment with 0.125 µg/ml AZM produced significant killing after 1 h (P < 0.01, Holm-Sidak test).

To determine whether inhibition of AZM transport impairs the killing of intraepithelial 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 (Fig. 4). Under these conditions, carnitine reduced the steady-state intracellular AZM concentration from 43.5 µg/ml to approximately 33.3 µg/ml (data not shown). Treatment for 1 h 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).

### 4.5 Discussion

The results obtained in this study demonstrate that human oral epithelial cell lines possess an active transport system that concentrates AZM and facilitates the 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 AZM concentration
ratios observed in the present study were approximately 3-fold higher than those reported for canine kidney, McCoy, and Hep-2 epithelial cells. The differences might be associated with the different origins of these epithelial cell lines and different levels of transporter gene expression. SCC-25 cells took up AZM at a Vmax 2-fold higher than that of 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 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 h 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 cells were 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 intraepithelial A. actinomycetemcomitans at the 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 the 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 h with 0.115 µg/ml moxifloxacin or 6.25 µg/ml doxycycline. Thus, it appears that moxifloxacin, doxycycline, and AZM can kill intraepithelial *A. actinomycetemcomitans* at concentrations near those attainable in GCF. One advantage of AZM is that its therapeutic levels in GCF are sustained for an unusually long time (≥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 at killing invasive infective *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.

### 4.6 References


(3) **Chen C, Slots J.** 1999. Microbiological tests for Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. Periodontol. 2000 **20:** 53-64.


Table 7. Kinetic constants for AZM transport by Smulow-Glickman and SCC-25 cells\textsuperscript{a}
\textsuperscript{a} $K_m$ and $V_{\text{max}}$ 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>
<thead>
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<th>Cell type</th>
<th>$K_m$ (µg/ml)</th>
<th>$V_{\text{max}}$ (ng/min/10\textsuperscript{6})</th>
<th>Cellular/extracellular concn ratio</th>
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<td>SG</td>
<td>198 ± 23.8</td>
<td>249 ± 13\textsuperscript{b}</td>
<td>23.7 ± 0.97\textsuperscript{b}</td>
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<tr>
<td>SCC-25</td>
<td>176 ± 10.5</td>
<td>486 ± 20.1</td>
<td>28.8 ± 1.53</td>
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Table 8. Inhibition of SCC-25 cell AZM transport by organic cations and probenecid

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<th>Agent</th>
<th>$K_m$ (µg/ml)</th>
<th>$V_{max}$ (ng/min/10^6)</th>
<th>Mechanism of inhibition ($K_i$ [mM])</th>
<th>Chemical classification</th>
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</thead>
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<td>486 ± 20.1</td>
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<td>Not applicable</td>
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<td>Quinidine</td>
<td>382 ± 2.0</td>
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<tr>
<td>Pyrilamine</td>
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<tr>
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<tr>
<td>l-Carnitine</td>
<td>314 ± 32</td>
<td>483 ± 46.5</td>
<td>Competitive (0.46 ± 0.05)</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)</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 2. Time course of AZM accumulation by cultured SCC-25 and SG epithelial cells at 37 degrees Celsius.

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 in the presence and absence of 1 mm carnitine. The intercepts are consistent with competitive inhibition.
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).
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 were 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).
Chapter 5

Azithromycin enhances phagocytic killing of *Aggregatibacter actinomycetemcomitans* Y4 by human neutrophils

5.1 Abstract

**Background:** *Aggregatibacter actinomycetemcomitans* (*Aa*) resists killing by neutrophils and is inhibited by azithromycin and amoxicillin. Azithromycin actively concentrates inside host cells, while amoxicillin enters by diffusion. The present study was conducted to determine whether azithromycin is more effective than amoxicillin at enhancing phagocytic killing of *Aa* by neutrophils.

**Methods:** Killing assays were conducted in the presence of either 2µg/ml azithromycin or 16µg/ml amoxicillin (equipotent against *Aa*). Neutrophils were loaded by incubation with the appropriate antibiotic. Opsonized *Aa* strain Y4 was incubated with the indicated antibiotic alone, with loaded neutrophils and antibiotic, or with control neutrophils (without antibiotic) at multiplicities of infection (MOI) of 30 and 90 bacteria per neutrophil.

**Results:** Neutrophil incubation with 2µg/ml azithromycin yielded an intracellular concentration of 10µg/ml. At an MOI of 30, neutrophils loaded with azithromycin failed to kill significantly more bacteria than control neutrophils during the 60 and 90 minute assay periods. At an MOI of 90, neutrophils loaded with azithromycin killed significantly more bacteria than either azithromycin alone or control neutrophils during 60 and 90 min incubations (P < 0.05), and killed significantly more bacteria after 90 minutes than the sum of the killing produced by azithromycin alone or neutrophils alone.
Neutrophils incubated with amoxicillin under identical conditions also killed significantly more bacteria than either amoxicillin alone or control neutrophils, but there was no evidence of synergism between amoxicillin and neutrophils.  

**Conclusion:** Neutrophils possess a concentrative transport system for azithromycin that may enhance killing of *Aa*. Its effects are most pronounced when neutrophils are greatly outnumbered by bacteria.

**5.2 Introduction**

Aggressive periodontitis (AgP) is an early-onset form of periodontitis characterized by periods of rapid periodontal destruction and a tendency to occur in families.\(^1\) The localized form of AgP is strongly associated with infections by the facultative species *Aggregatibacter actinomycetemcomitans*.\(^2\)-\(^4\) This pathogen possesses several traits that enhance its virulence, including the ability to invade gingival epithelial cells, produce a leukotoxin that kills neutrophils, and resist phagocytic killing.\(^5\)-\(^10\) Thus, it is difficult to completely eliminate *A. actinomycetemcomitans* from patients with AgP by mechanical removal of subgingival bacterial plaque alone.\(^11\) A widely used protocol for treatment of AgP combines an adjunctive regimen of amoxicillin (AMX) and metronidazole with mechanical plaque debridement.\(^12\) Although these two agents can enter cells by passive diffusion, neither is concentrated inside epithelium or neutrophils. This could limit this regimen’s effectiveness in killing intracellular *A. actinomycetemcomitans*.

Azithromycin (AZM) is taken up by gingival epithelial cells and is known to accumulate inside neutrophils.\(^13\),\(^14\) It is active against *A. actinomycetemcomitans* and its therapeutic levels in gingival crevicular fluid are sustained for at least 14 days after the last oral dose.\(^15\),\(^16\) Previous studies have shown that accumulation of AZM inside human gingival epithelial cells facilitates killing of intra-epithelial *A. actinomycetemcomitans*.\(^13\) When active transport of AZM was inhibited by a competitive inhibitor, there was a significant decrease in the killing of intra-epithelial bacteria. It is feasible that AZM accumulation by neutrophils could enhance phagocytic killing of *A. actinomycetemcomitans*. To address this hypothesis, we characterized neutrophil AZM
accumulation and examined its effects on killing of *A. actinomycetemcomitans*. The relationship between AZM accumulation and killing of intracellular bacteria was explored by determining whether azithromycin enhances phagocytic killing of *A. actinomycetemcomitans* to a greater extent than an equipotent concentration of AMX.

### 5.3 Materials and methods

Human neutrophils were isolated from blood collected from healthy volunteer donors. Informed consent was obtained through a protocol approved by the IRB. The blood was subjected to Ficoll-Hypaque density gradient centrifugation and dextran sedimentation. Residual erythrocytes were eliminated by hypotonic lysis. Afterwards, neutrophils were washed three times with Ca\(^{2+}\)/Mg\(^{2+}\)-free phosphate buffered saline and resuspended in Hank’s balanced salts solution (HBSS). Isolated cells were >99% neutrophils and >99% viable as assessed by trypan blue exclusion.

AZM transport was assayed by measuring changes in cell-associated radioactivity over time. Neutrophil suspensions were warmed to 37°C prior to incubation with \[^{3}H\]AZM (American Radiolabeled Chemicals, St. Louis, MO) at concentrations of 10µg/ml in time course assays and 8-50 µg/ml in kinetic assays to determine the Michaelis constant (K\(_{\text{m}}\)) and maximal velocity of transport (V\(_{\text{max}}\)). After the indicated time interval (3 minutes for kinetic assays and 1 to 20 minutes for assaying uptake time course), cell aliquots were withdrawn, layered over a mixture of canola oil/dibutylphthalate (3:10) and centrifuged for 35 seconds at 15,000 X g. Cell pellets were recovered and lysed for determination of AZM content by liquid scintillation counting. Intracellular AZM concentrations were calculated from measurements of intracellular AZM content and intracellular volume.

Pure cultures of *A. actinomycetemcomitans* strain Y4 (43718; American Type Culture Collection, Manassas, VA) were grown in brain–heart infusion broth (Becton, Dickinson and Company, Sparks, MD) at 37 °C in humidified air with 10% CO\(_2\). This strain is highly leukotoxic and belongs to the serotype most commonly isolated in patients with AgP. Bacteria were washed and opsonized at 37 °C for 30 minutes in HBSS containing 20% human serum pooled from ~200 donors (Sigma Chemical Co, St.
Louis, MO). Neutrophils were loaded for 15 minutes at 37 °C with either 2 m g/mL AZM (the lowest concentration typically measured in gingival crevicular fluid during a 2-week period after systemic administration\textsuperscript{16}; US Pharmacopeia, Rockville, MD) or 16 m g/mL AMX (US Pharmacopeia, Rockville, MD). Broth-dilution assays were used to confirm that these concentrations correspond to four times the MIC for \textit{A. actinomycetemcomitans} strain Y4.\textsuperscript{19} Control neutrophils were subjected to a similar incubation without antibiotic. Phagocytic killing assays were initiated by adding opsonized, prewarmed \textit{A. actinomycetemcomitans} suspensions to vials containing one of the following: 20% human serum in HBSS, either 2µg/ml AZM or 16µg/ml AMX in 20% serum, control neutrophils in 20% serum, or antibiotic-loaded neutrophils in 20% serum containing either 2µg/ml AZM or 16µg/ml AMX. Assays were conducted at MOI ratios of 30 and 90 bacteria per neutrophil. The incubation vials were slowly rotated end-over-end for 90 minutes at 37°C to promote phagocytosis. Under these conditions, neutrophil viability was >98% after 90 minutes. At the beginning of the incubation and every 30 minutes thereafter, aliquots were removed and diluted in sterile water to lyse the neutrophils. Following a second dilution, samples were spread on BHI agar plates. After incubation for 48 hours at 37°C in 10% CO\textsubscript{2}, surviving \textit{A. actinomycetemcomitans} colonies were counted to assess killing.

Repeated-measures analysis of variance (ANOVA) was used to evaluate the concentration-dependent differences in the intracellular AZM concentrations and percentages of bacteria killed in the phagocytic killing assays. These data were normally distributed and exhibited equal variance. The Holm-Sidak test was used for post hoc comparisons. Differences in the cellular/extracellular concentration ratios were examined by Friedman repeated measures ANOVA on ranks because the data were not normally distributed.

\textbf{5.4 Results}

At concentrations similar to those found in gingival crevicular fluid (2 to 10µg/ml), AZM was rapidly accumulated by neutrophils. Uptake exhibited Michaelis-Menten kinetics (Figure 5, inset), with an observed Michaelis constant of $197 \pm 12.8$ µg/ml and a maximum velocity of $59.9 \pm 5.52$ ng/min/$10^6$ cells. AZM uptake saturated
within 20 minutes (Figure 5), yielding intracellular concentrations approximately 5-fold higher than those in medium (Table 9). Over the range of 8º to 37º C, AZM transport was highly temperature-dependent (r = 0.975, data not shown).

AZM-loaded neutrophils produced more effective bacterial killing than control neutrophils or AZM alone. The magnitude of this effect was dependent on incubation time and the MOI. At an MOI of 30 bacteria per neutrophil, AZM-loaded neutrophils exhibited a small, but statistically significant enhancement of killing relative to control neutrophils at 30 minutes (P < 0.05, Holm-Sidak test), but failed to kill significantly more bacteria than controls at 60 and 90 minutes (Figure 6). AZM-loaded neutrophils killed significantly more bacteria than AZM alone at 30 and 90 minutes (P < 0.05). At an MOI of 90, AZM-loaded neutrophils killed significantly more bacteria than control neutrophils or AZM alone at 60 and 90 minutes (Figure 6, P < 0.05, Holm-Sidak test), but not at 30 minutes. At 60 minutes, the reduction in surviving CFU produced by AZM-loaded neutrophils (37.9%) was not significantly higher than the sum of the individual effects produced by AZM and neutrophils (P = 0.22, paired t-test). At 90 minutes, however, the reduction by AZM-loaded neutrophils (58.5%) was significantly more pronounced than the sum of the individual effects of AZM and neutrophils (P < 0.05).

Under the same conditions (MOI = 90), AMX-loaded neutrophils also killed significantly more bacteria than control neutrophils or AMX alone at 60 and 90 minutes (Figure 7, P < 0.05, Holm-Sidak test), but not at 30 minutes. At 60 minutes, the reduction in surviving CFU produced by AMX-loaded neutrophils (58.6%) was not significantly different from the sum of the individual effects produced by AMX and neutrophils. Similarly, at 90 minutes, the reduction by AMX-loaded neutrophils (65.6%) was not significantly different from the sum of the individual effects of AMX and neutrophils.

5.5 Discussion

The findings confirm that neutrophils take up AZM through an active transport system. The observed Michaelis constant and maximum velocity values for AZM transport were consistent with a previous report. Similar to clarithromycin (CLR), the
interaction of AZM with the transporter is of relatively low affinity. However, the transport velocity and the degree of intracellular concentration observed with AZM are lower than those of CLR.\textsuperscript{21} In spite of this, the results suggest that neutrophils exposed to 2μg/ml AZM while migrating through periodontal connective tissue toward the gingival crevice could accumulate an intracellular AZM concentration of approximately 10μg/ml. This is at least 5- to 40-fold higher than its MIC for most strains of \textit{A. actinomycetemcomitans} (0.25 - 2.0μg/ml) and 10- to 80-fold higher than its MIC for most strains of \textit{Porphyromonas gingivalis} (0.125 - 1.0μg/ml).\textsuperscript{15, 22} In contrast, the intracellular activity of AMX and other β-lactam antibiotics is relatively modest because it is not actively concentrated.\textsuperscript{23, 24} While the 16μg/ml concentration of AMX used in this study is as effective as 2μg/ml AZM with respect to inhibition of \textit{A. actinomycetemcomitans} strain Y4, this concentration is far above the normal therapeutic range. Treatment with AMX typically yields a concentration of 3 to 4μg/ml in gingival crevicular fluid.\textsuperscript{25}

The results suggest that AZM accumulation inside neutrophils enhances the phagocytic killing of \textit{A. actinomycetemcomitans}. At equipotent concentrations, killing by AMX alone appeared to be more effective than by AZM alone. At the higher MOI, however, the magnitude of killing by AZM-loaded neutrophils was significantly greater than the sum of the individual effects of AZM and neutrophils alone, while killing by AMX-loaded neutrophils appeared to be additive in nature. To put these findings into context, AMX is a time-dependent antibiotic that produces optimal killing at concentrations that are 2 to 4 times the MIC,\textsuperscript{26} while AZM is a concentration-dependent antibiotic with efficacy that directly related to peak concentration and area under the concentration curve.\textsuperscript{27} AZM concentrations of at least 10 times the MIC are required to produce optimal bacterial killing.\textsuperscript{28} Thus, the extracellular concentrations of AMX and AZM used in this study (which were both 4 times the MIC) were optimal for killing \textit{A. actinomycetemcomitans} with AMX, but sub-optimal for AZM. Intra-neutrophil accumulation of AZM at concentrations ≥10 times the MIC presumably played a role in increasing its activity against \textit{A. actinomycetemcomitans} and producing synergistic enhancement of killing by loaded neutrophils. Reinforcing the relationship between cell accumulation of AZM and its effect on killing of intracellular \textit{A. actinomycetemcomitans},
our laboratory recently reported that intracellular AZM accumulation also facilitates killing of invasive *A. actinomycetemcomitans* infections in cultured gingival epithelial cells. In that study, competitive inhibition of AZM transport into epithelial cells antagonized killing of intracellular *A. actinomycetemcomitans* laboratory strain Y4 and clinical strain SUNY 465 by AZM.

Because phagocytosis of *A. actinomycetemcomitans* by neutrophils is relatively inefficient, it is reasonable to doubt that intracellular accumulation of AZM could significantly enhance phagocytic killing. Along with resisting engulfment, however, *A. actinomycetemcomitans* also resists killing by neutrophils. Due in part to a weak oxidative burst response, the majority of ingested *A. actinomycetemcomitans* are still viable after one hour. AZM is protonated and trapped in acidic sub-cellular environments in neutrophils, including lysosomes. Under these conditions, prolonged exposure to high intracellular AZM concentrations has the potential to augment bacterial killing. It is important to note that AZM-loaded neutrophils were not consistently more effective at killing *A. actinomycetemcomitans* than control neutrophils in experiments that were run at an MOI of 30. Under these conditions, control neutrophils alone were capable of killing half of the bacteria over a period of 90 minutes. The beneficial effects of the system for intracellular AZM accumulation were mainly observed when neutrophils were presented with a greater number of bacteria than they could easily kill. It is possible that the serum used to opsonize the bacteria in this study could have influenced the degree of bacterial killing, since it may have contained antibodies that neutralized leukotoxicity. The serum was a commercial product that was pooled from a many human donors, and a large proportion of individuals with periodontitis express antibodies against whole *A. actinomycetemcomitans* as well as *A. actinomycetemcomitans* leukotoxin. While it is likely that these antibodies had similar effects on bacterial killing by the two antibiotics used in this study, it would have been useful to characterize the levels of antibodies in the serum.

A similar study has been conducted with CLR, a macrolide which has a shorter half-life than AZM, is more active against *P. gingivalis* and *Prevotella intermedia* and is comparably effective against *A. actinomycetemcomitans*. As with AZM, the most
significant differences in killing of *A. actinomycetemcomitans* by control and CLR-loaded neutrophils were observed at a relatively high MOI (100). One major difference was that the effects of CLR and neutrophils appeared to be additive rather than synergistic, despite the higher intracellular concentrations observed with CLR in neutrophils. In contrast to AZM, CLR is classified as a time-dependent antimicrobial agent, although it exerts post-antibiotic effects that are enhanced at higher concentrations. This difference in the pharmacodynamic properties of AZM and CLR could be partly responsible for their somewhat divergent effects on phagocytic killing of *A. actinomycetemcomitans*. Clinically, systemic CLR appears to enhance reduction of pocket depth and gain of clinical attachment resulting from non-surgical treatment of chronic periodontitis, but only one study has been conducted to date.

In addition to examining the killing of *A. actinomycetemcomitans* strain Y4, it would have been useful to include a clinical strain of *A. actinomycetemcomitans* in this study. Poly-N-acetylglucosamine, a matrix polysaccharide that helps *A. actinomycetemcomitans* resist phagocytic killing, is produced by most clinical strains, but not by strain Y4. It is reasonable to speculate that accumulation of antibiotic inside neutrophils could potentially have a more pronounced impact on killing of a clinical strain than it did on Y4. While this omission must be considered a limitation, the assay conditions used in the present study were representative of conditions that occur *in vivo*. A recent pharmacokinetic study found that AZM concentrations in gingival crevicular fluid can approach 10µg/ml and exceed 4 µg/ml for more than a week after the final oral dose. The study suggested that a standard regimen of AZM (initial dose of 500mg, followed by 250 mg every 24 hours for the next four days) provides tissue levels in excess of the 2 µg/ml concentration used in the current study for at least 15 days. Counts of *A. actinomycetemcomitans*, *P. gingivalis* and neutrophils recovered from AgP sites suggest that MOI ranges from 14:1 at a typical site to 167:1 at sites with the highest levels of *A. actinomycetemcomitans*, and up to 150:1 at sites with the highest levels of *P. gingivalis*. Thus, it is feasible that neutrophil killing of *A. actinomycetemcomitans* could be enhanced in AgP patients undergoing treatment with AZM, and conditions are also favorable for enhanced killing of *P. gingivalis*. Due to the
low prevalence of the localized form of AgP, there have been no randomized clinical trials to assess the effectiveness of AZM as an adjunct to periodontal treatment. There is evidence that adjunctive use of AZM can enhance the clinical response to non-surgical treatment of the generalized form of AgP as well as non-surgical treatment of severe chronic periodontitis in smokers.\textsuperscript{39,40} AZM has also been shown to enhance clinical and microbiological outcomes in the treatment of \textit{P. gingivalis}-associated chronic periodontitis.\textsuperscript{41} However, the improved clinical outcomes produced by adjunctive AZM have not always been associated with significant suppression of subgingival pathogens. Although some studies have shown that AZM suppresses periodontal pathogens,\textsuperscript{40,42} others either did not observe a significant adjunctive effect\textsuperscript{43} or did not conduct a statistical analysis of microbiologic data.\textsuperscript{44}

5.6 Conclusion

Neutrophils express an active transport system for azithromycin. Intracellular accumulation of azithromycin may enhance killing of \textit{A. actinomycetemcomitans}, especially under conditions in which neutrophils are greatly outnumbered by bacteria. The results of this study, in combination with a recent report that intracellular AZM accumulation can kill invasive \textit{A. actinomycetemcomitans} in gingival epithelial cells,\textsuperscript{13} suggest that adjunctive AZM can potentially enhance the elimination of \textit{A. actinomycetemcomitans} from patients with periodontitis.

5.7 References


Table 9. Intracellular accumulation of AZM by human neutrophils

<table>
<thead>
<tr>
<th>Extracellular AZM Concentration (μg/mL)</th>
<th>Intracellular AZM Concentration (μg/mL)*</th>
<th>Cellular/Extracellular Concentration Ratio†</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>9.82 ± 1.21</td>
<td>4.91 ± 0.60</td>
</tr>
<tr>
<td>5</td>
<td>23.8 ± 2.0</td>
<td>4.76 ± 0.40</td>
</tr>
<tr>
<td>10</td>
<td>49.1 ± 7.1</td>
<td>4.91 ± 0.70</td>
</tr>
<tr>
<td>20</td>
<td>94.8 ± 10.5</td>
<td>4.74 ± 0.52</td>
</tr>
</tbody>
</table>

Results are expressed as the mean and SD
*Within the column, there is a significant treatment effect (P<0.001, repeated measures ANOVA) and there is a significant difference in all pairwise comparisons (P<0.05, Holm-Sidak)
†Within the column, there are no statistically significant differences (P=0.39, Friedman repeated measures ANOVA on ranks)
Figure 5. Time course of accumulation of AZM by suspended neutrophils at 37 degrees Celsius

The data represent the mean and SD. Concentration of [$^3$H]-AZM is 10µg/ml. Inset: A representative Lineweaver-Burk plot of the initial phase of AZM transport by neutrophils.
Figure 6. Effect of AZM on killing of A. actinomycetemcomitans at multiplicities of infection of 30 and 90

Opsonized bacteria were added to tubes containing 20% human serum (bacteria only), 2 µg/ml AZM in 20% serum (bacteria + AZM), control neutrophils in 20% serum (bacteria + PMNs), or AZM-loaded neutrophils in 20% serum containing 2µg/ml AZM (bacteria, PMNs & AZM). Data are presented as the mean and SD of 5 experiments. At each time point, pair-wise comparisons that exhibited statistically significant differences (as determined by the Holm-Sidak post-hoc test) are denoted by identical letters.
Studies were conducted under the same conditions as the lower panel of Figure 6, except that AMX (16 µg/ml) was substituted for AZM. Data are presented as the mean and SD of 5 experiments. At each time point, pair-wise comparisons that exhibited statistically significant differences (as determined by the Holm-Sidak test) are denoted by identical letters.
Relative effectiveness of azithromycin in killing intracellular Porphyromonas gingivalis

6.1 Abstract

Objective and Background: Invasive infections by Porphyromonas gingivalis are associated with persistent periodontal attachment loss and can be difficult to eliminate by scaling and root planing. Azithromycin (AZM) inhibits P. gingivalis and is actively accumulated by most human cells. We used an in vitro infection model to compare the effectiveness of AZM in killing intracellular P. gingivalis to the commonly-used combined regimen of amoxicillin (AMX) and metronidazole (MET).

Methods: Transport of [3H]-AZM by human gingival fibroblasts was characterized. Monolayers of Smulow-Glickman gingival epithelial cells or gingival fibroblasts were infected with P. gingivalis (either strain 33277 or W83). After extracellular bacteria were eliminated by treatment with teicoplanin, infected cells were treated with therapeutic concentrations of AZM, AMX, or AMX+MET. Viable intracellular bacteria were released by cell lysis and plated on blood agar for enumeration. Antimicrobial activity against planktonic P. gingivalis was also evaluated.

Results: While survival of intra-epithelial P. gingivalis 33277 was not significantly different after treatment with the three regimens, survival in infected fibroblasts was significantly lower after AZM treatment (65.9±5.5%) compared to AMX (92.2±3.5%) or AMX+MET (79.8±5.2%, p<0.01). Carnitine, a competitive inhibitor of AZM transport, reduced the killing effect of AZM by ~55% (p<0.05). Survival of intra-fibroblast P. gingivalis W83 was also significantly lower after AZM treatment compared to the other
regimens (P< 0.05). At therapeutic concentrations, AZM was significantly more active against intracellular *P. gingivalis* than against planktonic *P. gingivalis* (p < 0.0083).

**Conclusions:** Human gingival epithelial cells and fibroblasts possess a transport system that accumulates AZM and enhances elimination of intracellular *P. gingivalis*. Compared to the combination of AMX and MET, AZM was equally effective against intra-epithelial *P. gingivalis* 33277 and significantly more effective against both strains of *P. gingivalis* from infected gingival fibroblasts. The results suggest that AZM could be a reasonable alternative to the regimen of AMX and MET for periodontal patients who can’t take these agents due to allergy, known side effects, or compliance issues.

6.2 Introduction

Periodontitis is an infectious disease resulting in inflammation within the supporting tissues of the teeth and progressive loss of attachment and bone (1). It reportedly affects 47.2% of US adults aged 30 years and older (2). In periodontitis, polymicrobial communities transition from physiologically compatible commensal bacteria to pathogenic entities with the presence of some keystone pathogens. The complex interactions among members of microbial communities and between the communities and host immune system can be explained through a polymicrobial synergy and dysbiosis model (3). Control of subgingival bacterial biofilms through mechanical treatment (scaling and root planing) usually halts the progression of periodontal breakdown, but the responses are not favorable in some patients. Some keystone periodontal pathogens, including *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, have the ability to invade pocket epithelium and underlining connective tissue, evading the host immune system and lingering in the periodontal tissue. This could partly explain the unresponsiveness and recurrence of periodontitis (4). Although adjunctive systemic antibiotics are not routinely indicated in periodontal therapy, it is reasonable to use antibiotics that can cross cell membranes to control intracellular infections (5).

*P. gingivalis* is a gram-negative, non-motile, obligate anaerobe which exhibits a strong association with chronic periodontitis (6, 7). Its ability to invade gingival
epithelium and fibroblasts makes it difficult to eliminate by conventional therapy (8, 9). Its major virulence factors, including gingipains, fimbriae, lipopolysaccharide, and capsules, have also been attributed to dysregulation of the host immune-inflammatory responses (10). *P. gingivalis* has been used as a bacterial marker for progression of periodontitis (11).

Azithromycin (AZM) is a macrolide antibiotic derived from erythromycin. Its properties include broad-spectrum activity against most species of periodontal pathogens, a plasma half-life of over 65 hours, ability to be actively taken up by mammalian cells, and more sustained concentrations in gingival tissues and gingival crevicular fluid than in serum (12-14). Its antimicrobial activity against *P. gingivalis* has been confirmed in *in vitro* and clinical studies (15-18). Adjunctive use of systemic AZM with scaling and root planing results in significantly more reduction of probing depth and gain of clinical attachment in patients with chronic or aggressive periodontitis (18-20).

The combination of amoxicillin (AMX) and metronidazole (MET) is the most commonly used antibiotic regimen in periodontal therapy and is effective in the treatment of both chronic and aggressive periodontitis (21, 22). Neither AMX nor MET is concentrated inside human cells (23, 24). Our laboratory has previously shown that intracellular AZM accumulation helps eliminate *Aggregatibacter actinomycetemcomitans* from infected gingival epithelial monolayers (25). Since no previous study has directly compared AZM to the combined regimen, we used a similar approach to test the hypothesis that AZM is as effective at killing intracellular *P. gingivalis* in gingival fibroblasts as AMX plus MET. The association between intracellular AZM accumulation and intracellular activity was also examined.

### 6.3 Materials And methods

**Cell culture:** Smulow-Glickman (SG) gingival epithelial cells derived from human attached gingiva (26) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA). A human gingival fibroblast strain harvested as previously described (27) was cultured in minimal essential medium
(Invitrogen) containing 2 mM L-glutamine and 10% heat-inactivated FBS. Both cell lines were fed every 3 days and cultured to confluent monolayers at 37°C in the presence of 5% CO₂ until a confluent monolayer was formed.

**Bacterial culture:** *P. gingivalis* 33277 (American Type Culture Collection, Manassas, VA) and a well-characterized laboratory strain (W83) were grown in Trypticase soy broth (Becton, Dickinson and Company, Sparks, MD) supplemented with 1 mg/ml yeast extract (Gibco, Grand Island, NY), 5 µg/ml hemin (Sigma-Aldrich, St. Louis, MO) and 1 µg/ml menadione (Sigma-Aldrich) at 37°C in an anaerobic chamber (28).

**Antibiotics:** All targeted antibiotics, including AZM, AMX, MET, and moxifloxacin (MXF), were from Sigma-Aldrich. Teicoplanin was acquired from Selleck Chemicals LLC (Houston, TX). The MIC of each antibiotic against planktonic *P. gingivalis* were determined by broth dilution methods (29). Briefly, overnight cultures of *P. gingivalis* were resuspended in TSB supplemented with yeast extract, hemin, and menadione to an optical density of 0.001 at 600 nM, which corresponds to 10⁶ CFU/ml. *P. gingivalis* were then cultured anaerobically for 48 hours to determine the lowest concentration which prevents visible growth of bacteria (MIC).

**Assays of AZM transport:** Confluent gingival fibroblast monolayers were washed with Hank’s balanced salt solution (HBSS; Invitrogen), harvested by brief treatment with 0.25% trypsin EDTA (Invitrogen), and suspended in HBSS at a density of 10⁶ cells/ml. AZM transport was assayed by measuring changes in cell-associated radioactivity over time (30). Aliquots of cell suspension were incubated at 37°C with [³H]-AZM (American Radiolabeled Chemicals, St. Louis, MO) at a concentration of 10 µg/ml for time course assays and at 10 to 50 µg/ml for kinetic assays to determine the Michaelis constant (*Kₘ*) and maximal velocity of transport (*Vₘₐₓ*). After the indicated interval (1 to 30 min for uptake time course assays 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-dibutyl phthalate (3:10), and centrifuged for 30 sec at 15,000 x g in a microcentrifuge (31). 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 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 [\(^{14}\)C]-inulin (1 µCi/ml; PerkinElmer, Waltham, MA) (32).

To observe the efflux of AZM, gingival fibroblasts were loaded to a steady-state intracellular AZM concentration by incubation for 20 min at 37°C in HBSS containing 10 g/mL [\(^{3}\)H]-AZM. To trigger efflux of intracellular AZM stores, we abruptly diluted AZM in the extracellular medium 11:1 with 37°C HBSS. The decrease in intracellular AZM concentration was monitored for 60 min.

**In vitro infection model:** The protocol of infection assays was modified from Lamont et al (28). In brief, confluent SG epithelial cell and gingival fibroblast monolayers in 24-well culture plates were washed twice with HBSS. *P. gingivalis* from overnight culture were harvested, resuspended in cell culture medium specific to the corresponding host cell lines, and laid over monolayers to infect at a 90 bacteria to 1 host cell ratio for 90 min. Afterward, extracellular bacteria were removed by HBSS washes and treatment with 100 µg/ml teicoplanin for 1 h. The monolayers were again washed four times with HBSS and an aliquot of the final wash was plated on reduced blood agar (Anaerobic systems, Morgan Hill, CA) to ensure that HBSS washes and treatment with teicoplanin removed viable extracellular bacteria (33). Infected cell monolayers were then cultured in the presence of AMX (4 µg/ml), AMX (4 µg/ml) + MET (10 µg/ml) and AZM (8 µg/ml), which correspond to therapeutic concentrations attainable in gingival crevicular fluid, for 4 h (34). As controls, infected monolayers were also cultured under identical condition in the absence of any antibiotic, and in the presence of 3.2 µg/ml moxifloxacin (MXF) (33). At the end of incubation, monolayers were washed four times with HBSS and lysed in sterile water to release intracellular *P. gingivalis*. Dilutions of the lysate were plated on reduced blood agar plates for the enumeration of surviving CFU. Data were expressed as a percentage of the colonies recovered from the controls without antibiotics.

To determine whether the intracellular accumulation of AZM enhances the killing of intracellular *P. gingivalis*, antibiotic efficiency against planktonic bacteria was tested under similar conditions and compared to that against intracellular bacteria. In addition,
an AZM transport inhibitor (L-carnitine) was added to the epithelial model to evaluate its influence on killing of *P. gingivalis* (25).

6.4 Results

**AZM transport by gingival fibroblasts:** Gingival fibroblasts rapidly took up AZM, reaching the point of saturation after 15 min (**Fig 8**). When gingival fibroblasts were incubated in medium containing clinically attainable concentrations of AZM for 20 min, the steady-state intracellular concentrations were at least 10-fold higher than the extracellular concentrations (**Table 10**). The observed AZM transport activity exhibited Michaelis-Menten kinetics, and kinetic analysis indicated the gingival fibroblasts internalized AZM with an estimated $K_m$ of 262 ± 12.0 µg/ml and a maximum velocity ($V_{max}$) of 394 ± 39.0 ng/min/10^6 cells (**Fig 8 inset**). The efflux assays indicated that AZM transport is bi-directional. Once the AZM concentration in the medium was diluted 11-fold, gingival fibroblasts lost approximately 33% of their AZM content within 3 min and almost 60% after 20 min. Thereafter, the rate of efflux decreased dramatically. At the end of the 60-min observation, the gingival fibroblasts retained approximately 30% of their original steady-state AZM content (**Fig 9**).

**Killing of *P. gingivalis* 33277 by selected antibiotics:** After 4-hour incubation under conditions to similar to the infection assays, AMX and AMX + MET produced comparable killing on planktonic *P. gingivalis* 33277 (~19% and ~22% of the control, respectively). AZM showed little effect while the positive control, MXF, killed more than 99% of planktonic bacteria. The differences between AZM and the other regimens were statistically significant (p < 0.01, Holm-Sidak test) (**Fig 10**).

All tested antibiotic regimens showed different levels of killing against intra-fibroblast *P. gingivalis* 33277 (**Fig 10**). AZM killed approximately 34% of the *P. gingivalis* in infected gingival fibroblasts, while AMX and AMX + MET killed ~8% and ~20%, respectively. Approximately 99% of the intra-fibroblast bacteria were killed by MXF over the same period. The differences among all regimens were statistically significant (p < 0.05, Holm-Sidak test). It appeared that AZM was significantly more effective against intra-fibroblast *P gingivalis* 33277 than against planktonic bacteria.
(~34% vs ~0.5%, p < 0.0083, $t$ test, after adjustment), but this phenomenon was not observed from either AMX or AMX + MET.

As for intra-epithelial *P. gingivalis* 33277, all tested antibiotic regimens showed significant suppression of intracellular bacteria (p < 0.05, Holm-Sidak test). Killing of intra-epithelial *P. gingivalis* was ~43%, ~38%, and ~33% of the control CFU after treatment with AZM, AMX + MET, and AMX, respectively (Fig 10). Meanwhile, MXF killed more than 99% of intra-epithelial *P. gingivalis*. The differences among AMX, AMX + MET, and AZM failed to reach statistical significance (p > 0.05). Only AZM exhibited significantly greater killing of intra-epithelial *P. gingivalis* relative to planktonic *P. gingivalis* (p < 0.0083, $t$ test, adjusted).

A separate series of transport assays in which 2 mM L-carnitine and 4 µg/ml AZM were co-incubated with SG epithelial cells for 4 h indicated carnitine reduced the steady-state intracellular AZM concentration from 92.0 µg/ml to ~68.5 µg/ml (data not shown). Carnitine alone reduced approximately 10% of intra-epithelial *P. gingivalis* 33277, whereas AZM alone killed ~30% of the bacteria. In the presence of carnitine, more than half of the killing effect produced by azithromycin was reversed (p < 0.05, Holm-Sidak test, data not shown). The difference in effects produced by carnitine alone and in combination with AZM was not statistically significant (p > 0.05).

**Killing of *P. gingivalis* W83:** The levels of inhibition on planktonic *P. gingivalis* W83 generated from AMX and AMX+ MET were comparable (~18% and ~19%, respectively). In contrast to its activity against the 33277 strain, AZM killed ~53% of planktonic *P. gingivalis* W83, which is significantly more active than the other two regimens (p < 0.01, Holm-Sidak test). The positive control (MXF) killed approximately 90% of bacteria (Fig 4).

A similar trend was observed with killing of intra-fibroblast *P. gingivalis* W83. AZM eliminated approximately 70% of the control *P. gingivalis* W83, whereas AMX and AMX + MET had no significant effect on intracellular bacteria (~2% and ~4%, respectively, Fig 11). The antimicrobial activity of AZM against intra-fibroblast W83 was significantly greater than that of AMX or AMX + MET (p < 0.01, Holm-Sidak test).
In addition, AZM produced significantly stronger antimicrobial activity against intra-fibroblast W83 than against their planktonic counterparts (p < 0.0167, t-test, adjusted).

6.5 Discussion

Our data indicate that human gingival fibroblasts take up and accumulate relatively high intracellular concentrations of AZM through an active transport system. When gingival fibroblasts were cultured in medium containing 5 µg/ml AZM, the steady-state intracellular AZM concentration was approximately 55.8 µg/ml. Intracellular stores of AZM appears to move out of fibroblasts when the extracellular concentration drops, which could help maintain high concentrations in tissue fluid. SG gingival epithelial cells used in this study also have an active transport system that exhibits similar characteristics and concentrates azithromycin more than 20 times inside (25). Human neutrophils also have an active transport system for AZM (35, 36). To date, only one pharmacokinetic study has been conducted in human fibroblasts (37); however, some of the observed characteristics in the current study were different from those found in human skin fibroblasts. AZM uptake by human skin fibroblasts was reportedly continuous over a 3-day period, leading to extracellular/cellular concentration ratios of 174 and 3738 after 1 hour and 72 hours, respectively. The differences could be partly explained by different cell origins and incubation conditions (adherent versus suspended cells). It has been reported that AZM concentration in lysosomes changes lysosomal pH and function in phagocytes (38). The maximal non-cytocidal concentration of AZM for a human gingival epithelial cell line NDUSD-1 was reported to be 10 µM (39). It is reasonable to expect that uptake reaches a point of saturation once intracellular AZM starts to affect cellular functions. Given their large numbers in gingival connective tissue, the ability to take up and retain AZM could allow gingival fibroblasts to function as “reservoirs” that maintain high tissue concentrations (40, 41).

The antimicrobial activities of the targeted antibiotics were compared under similar aerobic conditions. Although all antibiotics were active against *P. gingivalis* 33277, as judged by the MIC values tested in our laboratory (<0.03, <0.015, 0.5 µg/ml for AMX, MET, and AZM, respectively), their activity against planktonic bacteria was
rather modest. This could be partially explained by the experimental conditions. When exposed to normal aerobic tissue culture atmosphere, *P. gingivalis*, an obligate anaerobe, typically synthesizes proteins and enzymes such as heat shock proteins and superoxide dismutase for survival (instead of proteins for multiplication) (42). In addition, the medium used for these assays did not contain hemin and menadione and was less supportive of bacterial growth (43). AMX, which inhibits peptidoglycans cross-linking (44), may not work efficiently when bacteria are not actively replicating. MET is a prodrug that has no antimicrobial effects before its reduction by anaerobic enzyme systems such as pyruvate:ferredoxin oxidoreductase and NADPH nitroreductases (45, 46). Under our experimental conditions, there may not have been sufficient reduced intermediate to interact effectively with bacterial DNA. AZM inhibits bacterial growth by binding to the 50S ribosomal subunit of the bacterial ribosome (47). It is interesting that we didn’t observe any antimicrobial activity against planktonic *P. gingivalis*, considering its potential to arrest synthesis of enzymes and proteins that help *P. gingivalis* survive. In contrast, MXF (3.2 µg/ml) almost completely eliminated planktonic *P. gingivalis* within 4 hours. Moxifloxacin is a fluoroquinolone antibiotic and exerts its bactericidal effect mainly through inhibition of DNA gyrase, which is required for DNA replication, transcription and repair (48). The effective and efficient killing by MXF is consistent with a previous study (49).

Our results suggest that AZM is more effective at killing intra-fibroblast *P. gingivalis* 33277 than AMX or AMX + MET. Compared to its weak activity against planktonic *P. gingivalis*, AZM appears to benefit substantially from its cellular accumulation by gingival fibroblasts. AZM exhibits concentration-dependent killing kinetics, in which efficacy can be predicted by the area under the concentration-time curve over 24 h (AUC) divided by the MIC (50). The active transport system of fibroblasts concentrates AZM, leading to an elevated AUC/MIC ratio and greater bacterial inhibition. In contrast, the intracellular concentrations of AMX and MET have been shown to be equal to or less than the extracellular concentrations (23, 24). This, along with environmental limitations mentioned in the previous section, could explain why AMX and AMX + MET did not exhibit improved activity against intra-fibroblast *P.
In general, the results observed with *P. gingivalis* W83 were similar to those seen with strain 33277, except for the greater suppression produced by AZM. Comparison of the antimicrobial activities against planktonic and intra-fibroblast W83 indicated that cellular accumulation enhanced killing of bacteria.

Our study indicated that active transport by SG gingival epithelial cells enhanced AZM’s antimicrobial activity against intracellular *P. gingivalis* 33277, and this enhancement could be reversed by an inhibitor of the transporter. Based on our previous study, some organic cations including quinidine and L-carnitine competitively inhibit azithromycin transport by oral and gingival epithelial cells (25). The relationship between intracellular AZM concentrations and antimicrobial activity is also evident in other studies. Addition of L-carnitine reduced killing of intracellular *A. actinomycetemcomitans* to 54% of that produced by AZM alone in SG gingival epithelial cells (25). In a macrophage model, verapamil (an inhibitor of P-glycoprotein efflux pump) increased intracellular AZM concentrations by ~2.4 fold and enhanced the activity against *Listeria monocytogenes* and *Staphylococcus aureus* when low extracellular concentrations were used (51). Human neutrophils loaded with AZM exhibited an antimicrobial effect significantly greater than the sum of suppression produced by AZM or neutrophils alone. This benefit was most pronounced when bacteria vastly outnumbered neutrophils (52).

It is interesting to observe differences in the relative effectiveness of AZM compared to the other antibiotics. AZM is more effective against intra-fibroblast *P. gingivalis*, but is only equally effective in the epithelial cell model. We speculate that gingival epithelial cells may be a more hospitable environment to *P. gingivalis* than gingival fibroblasts, and AMX and AMX + MET may work better due to increased bacterial activities. There could be several factors that, alone or together, make epithelial cells a better environment in which to survive and replicate. It is evident that epithelial cells provide some levels of protection compared to the extracellular environment (53, 54). The host anti-oxidative mechanisms could potentially create a permissive environment (55). In addition, *P. gingivalis* may respond to epithelial cells and fibroblasts differently after their initial contact and invasion into these cells. Genes involved with the oxidative stress response and heat shock proteins are up-regulated.
significantly during infection of HEp-2 human epithelial cells (54). Although similar studies have not previously been conducted in fibroblasts, significant differences in the behavior of *P. gingivalis* could potentially make them more susceptible to antibiotics in epithelial cells.

The limitations of this study are mainly related to differences between our experimental model and *in vivo* conditions. Since periodontitis is a response to polymicrobial biofilm, our focus on a single pathogen in a planktonic state is an oversimplified approach. The aerobic culture conditions used in the model were adjusted to optimize the viability of human cells, but these conditions limit the growth of *P. gingivalis*. For this reason, the in-vitro model could only be used for relatively brief periods of antibiotic treatment.

The role cell invasion by bacteria plays in the pathogenesis of periodontitis is controversial. While the ability of bacteria to invade host cells and spread from cell to cell has been proposed as a possible mechanism in periodontal and cardiovascular diseases (56-58), the effectiveness of MET (alone or in combination with AMX) in clinical trials suggests that it is not necessary to target intracellular bacteria to obtain an adjunctive benefit. The results of this study do not detract from the effectiveness of the combined regimen of MET and AMX observed in clinical studies. Although the overall antimicrobial activity of AZM against *P. gingivalis* in our study is modest (killing only 20 to 40% of control CFU), it is comparable to the activity of MET, clindamycin, and doxycycline reported in a previous study (33). The favorable clinical and microbiological outcomes in clinical studies may be a result of persistent antimicrobial activity over time. In patients with *P. gingivalis*-associated periodontitis, adjunctive use of azithromycin leads to significant clinical improvement compared to scaling and root planing alone, and the suppression on *P. gingivalis* was evident up to 6 months (18).

### 6.6 Conclusions

Our data suggest that human gingival fibroblasts possess an active transport system that accumulates AZM at intracellular concentrations at least 10-fold higher than those in the extracellular environment. This accumulation enhances the antimicrobial activity of
AZM against intracellular *P. gingivalis* and makes it more effective than than the combination of MET and AMX in eliminated the bacteria from infected gingival fibroblasts. In gingival epithelial cells, AZM was equally effective in eliminating *P. gingivalis* 33277. It is reasonable to prescribe AZM instead of the MET/AMX regimen in periodontal applications for patients who are allergic to β-lactam drugs or are hypersensitive to the side effects of MET or AMX. In theory, it should be easier to obtain a high degree of compliance with AZM compared to the combined regimen. Within the limitation of this study, the data suggest that AZM is a reasonable alternative to AMX+MET when prescribing a systemic antibiotic to treat *P. gingivalis*-associated periodontitis.

### 6.7 References


<table>
<thead>
<tr>
<th>Extracellular concentration (µg/ml)</th>
<th>Gingival epithelium ¶</th>
<th>Gingival fibroblast</th>
</tr>
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<tbody>
<tr>
<td>0.5</td>
<td>N/A</td>
<td>10.64 ± 1.99</td>
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<tr>
<td>2</td>
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<td>10</td>
<td>18.34 ± 2.44</td>
<td>10.67 ± 1.85</td>
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Table 10. AZM cellular/extracellular concentration ratios in SG epithelial cells and gingival fibroblasts

Intracellular AZM content was determined after incubation with \( [^3H]-AZM \) for 20 min. All data are expressed as the mean ± SEM of 3 experiments. ¶ denotes data adapted from a previous publication with permission (22).
Figure 8. Time course of AZM uptake in gingival fibroblasts

Transport was initiated by the addition of $[^3]$H-AZM to aliquots of suspended cells. Changes in cell-associated radioactivity over time were subsequently determined by liquid scintillation counting. All data are expressed as the mean ± SEM of 6 experiments. The Inset is as representative Lineweaver-Burk plot of the initial phase of AZM transport by the gingival fibroblasts.
Figure 9. Efflux of AZM from loaded gingival fibroblasts

Gingival fibroblasts were loaded to steady state by incubation in medium containing 10 µg/ml [³H]-AZM for 20 min. Efflux was triggered by diluting extracellular antibiotic medium 11 fold with 37°C medium. All data are expressed as the mean ± SEM of 3 experiments. For references, 100% content corresponds to 60 ng/10⁶ cells.
Figure 10. Killing of planktonic, intra-epithelial, and intra-froblast *P. gingivalis* 33277 by AZM

SG epithelial cell or gingival fibroblast monolayers infected by *P. gingivalis* 33277, or planktonic bacteria suspended in medium were treated with 8 µg/ml AZM, 4 µg/ml AMX, 4 µg/ml AMX + 10 µg/ml MET, or 3.2 µg/ml MXF. Monolayers incubated in antibiotic-free medium were used as negative control. Intracellular *P. gingivalis* were released by cell lysis, plated on reduced blood agar and enumerated. Data were presented as percentage of the colonies recovered from the negative controls. The data represent the mean ± SEM of at least 4 experiments. Within each cluster, significantly different effects among regimens are denoted by different letters (P< 0.05, Holm-Sidak test).
Figure 11. Killing of planktonic and intra-fibroblast *P. gingivalis* W83 by AZM

Data were presented as percentage of the colonies recovered from the controls. The data represent the mean ± SEM of at least 7 experiments. Within each cluster, significantly different effects among regimens are denoted by different letters (P< 0.05, Holm-Sidak test).
Chapter 7

Conclusions and future research directions

7.1 Conclusion

In Chapters 4, 5, and 6, in vitro transport studies demonstrated that oral epithelial cells, gingival fibroblasts, and neutrophils take up azithromycin through an active transport system. Based on the common characteristics of the transport process, it appears that this transport system plays a conserved role in different types of human cells. The uptake process usually reaches a point of saturation within 20 minutes and results in a high degree of intracellular accumulation, with concentrations substantially higher than the extracellular concentration. The transport system maintains a fine equilibrium between the intracellular and extracellular concentrations in each cell and allows the release of intracellular stores of azithromycin when the extracellular concentrations decrease. Transport studies of epithelial cells further indicate that azithromycin transport is inhibited by agents with similar molecular features through a competitive inhibition mechanism. As discussed below, azithromycin transport appears to provide clinical benefits in several ways.

Fibroblasts are the major cellular compartment in healthy gingiva, and possession of an active transport systems allow fibroblasts to serve as azithromycin reservoirs. Together with other cell types, fibroblasts in different organs actively take up azithromycin, leading to increased tissue distribution. When azithromycin concentrations in serum and interstitial fluid decrease due to normal metabolic and excretive processes, azithromycin transporters mediate a gradual efflux of intracellular azithromycin to maintain the concentrations in gingival tissue and gingival crevicular fluid. This is consistent with results from Chapter 3, which show that azithromycin concentrations in
gingival crevicular fluid are significantly higher and more sustained than those in serum. While azithromycin concentrations in gingival crevicular fluid did not exhibit statistically significant changes from day 2 to day 7 after the initial dose, serum concentrations on day 7 were only 20% of those on day 2. The sustained concentrations in gingival crevicular fluid imply that azithromycin’s antimicrobial effects persist for extended periods of time after administration. This property, along with a long half-life, facilitates a once-a-day dosage regimen that helps improve patient compliance.

Similar results were observed in the study included in the Appendix, in which the concentrations of azithromycin in gingival crevicular fluid were several times higher than the minimal concentrations required to inhibit many periodontal pathogens at a time point 2 weeks after the final dose. The study also demonstrated that azithromycin perfusion into the gingival crevice is increased in the presence of gingivits. A direct contributing factor to this phenomenon is neutrophils and their azithromycin transport system. Circulating neutrophils take up azithromycin in blood and help to deliver azithromycin to inflammatory sites.

It is evident that the volume of gingival crevicular fluid is associated with the level of inflammation in gingiva. A previous study from our research group demonstrated that azithromycin decreases the content of inflammatory mediators, including IL-1β, IL-8, TNF-α, and VEGF in gingival crevicular fluid and induces a decrease in gingival crevicular fluid volume. Our pharmacokinetic studies show a consistent reduction in gingival crevicular fluid volume in both clinically healthy and inflamed tissues.

Another benefit of intracellular accumulation of azithromycin is enhanced intracellular antimicrobial activity. The in vitro infection studies in Chapter 4 suggested that azithromycin, at concentrations attainable in gingival crevicular fluid (8 µg/ml), exhibited significantly more effective killing of intraepithelial A. actinomycetemcomitans SUNY 465 than amoxicillin, which does not concentrate inside cells. Even at extracellular concentrations below its minimal inhibitory concentration (0.125 µg/ml), intraepithelial accumulation of azithromycin produced significant killing of invasive A. actinomycetemcomitans. Addition of competitive inhibitors of azithromycin transport
reduced not only intracellular concentrations of azithromycin but also the killing of intracellular \textit{A. actinomycetemcomitans} SUNY 465 in infected gingival epithelial cells. In Chapter 6, similar results were observed that impeded azithromycin transport by \textit{L}-carnitine resulted in attenuated killing of intra-epithelial \textit{P. gingivalis} 33277. Compared to its effects on extracellular, planktonic \textit{P. gingivalis}, azithromycin was significantly more effective in killing intra-epithelial and intra-fibroblast \textit{P. gingivalis} once it concentrated inside host cells. The experiments in Chapter 5 indicated that, compared to control neutrophils, neutrophils loaded with either amoxicillin or azithromycin enhanced the phagocytic activity, especially when neutrophils were outnumbered by \textit{A. actinomycetemcomitans} Y4; however, azithromycin produced a synergistic effect on bacterial killing while amoxicillin at equipotent concentrations showed only an additive effect.

In summary, the pharmacokinetic and mechanistic investigations provide more insight into the role of azithromycin in periodontal therapy. The pharmacological properties make azithromycin an ideal choice against intracellular infections by \textit{A. actinomycetemcomitans} and \textit{P. gingivalis}. Our studies suggest that azithromycin is a good alternative to the commonly prescribed amoxicillin/metronidazole combined regimen in treatment of periodontitis.

\subsection*{7.2 Future Direction}

In our studies, azithromycin appears to be more effective against invasive infections by \textit{A. actinomycetemcomitans} than amoxicillin. This pathogen is most strongly associated with localized aggressive periodontitis and there have been no clinical studies to assess the clinical, microbiological, and immunological effects of adjunctive systemic azithromycin in combination with scaling and root planing in this specific variant of periodontitis. Our research project provides a rational basis to pursue a randomized clinical trial in patients with localized aggressive periodontitis. In addition, a randomized clinical trial with parallel arms to compare azithromycin to other antibiotic regimens in
patients who require adjunctive antibiotics (such as recurrent periodontitis) could provide more evidence and shape the guidelines for clinical practice.

In analogy to the breakdown of supporting tissues around natural teeth in periodontitis, the destructive, inflammatory condition affecting their counterparts around dental implants is termed as peri-implantitis. Some studies indicate that peri-implant tissues tend to exhibit a higher pro-inflammatory status than periodontal tissues under clinically healthy conditions and during wound healing.\textsuperscript{3,4} It is also evident that the apical extension of the inflammatory lesions was more pronounced in peri-implantitis than in periodontitis.\textsuperscript{5} Bacterial plaque is believed to be a critical factor for development of peri-implant inflammation, which leads to loss of attachment and bone, and implant failure.\textsuperscript{6} Because bacterial plaque colonizes the surfaces of dental implants and elicits host responses in patterns similar to those seen in periodontitis, most of treatment modalities proposed for peri-implantitis follow comparable concepts in periodontal therapy. Various treatment options have been evaluated, but it is unclear which is the most effective approach. While antibiotics have played a significant role in periodontal therapy, current scientific evidence supporting use of locally or systemically administered antibiotics in the treatment of peri-implantitis is insufficient.\textsuperscript{7} Due to the localized nature of peri-implantitis, a few studies using locally delivered antibiotics were conducted and demonstrated moderate improvement. To date, there have been no randomized clinical trials of systemic antibiotics in patients with peri-implantitis. Based on our data and previous studies, azithromycin may have the potential to produce both antimicrobial and anti-inflammatory benefits in treatment of peri-implantitis. It appears that well-designed randomized clinical trials evaluating effects of adjunctive systemic azithromycin in nonsurgical and surgical treatment of peri-implantitis could help meet the current needs in this field.

The immuno-modulatory properties of azithromycin have been valuable in disease and conditions distinct from infection, but it is unclear whether this property could be beneficial in treatment of periodontitis. The observed reduction in gingival crevicular fluid volume in our studies (Chapter 3 and the Appendix) is a sign of azithromycin’s immuno-modulatory effect on cells in gingiva. Host modulation as a
therapeutic strategy in the management of periodontitis has been advocated for more than 15 years,\textsuperscript{8} and some agents such as sub-antimicrobial dose doxycycline and resolvins have shown promising results.\textsuperscript{9} To date, the mechanisms by which azithromycin exerts its host immuno-modulatory effects have not been fully elucidated. Mechanistic investigations using cells in the gingiva could potentially enhance our management of persistent, over-reactive periodontal inflammation and facilitate future pharmaceutical design of host modulation agents. Although long-term use of low dose azithromycin has been evaluated in the management of chronic pulmonary disease, drug resistance is always a critical problem that comes with prolonged use of antibiotics. To overcome the potential resistance issue, the evaluation of sub-antimicrobial dose azithromycin and chemically modified azithromycin in host modulation could also be valuable.

7.3 References


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Appendix: Effect of gingivitis on azithromycin concentrations in gingival crevicular fluid (Master thesis of Nidhi Jain, DDS, MS)

ABSTRACT

Aim: Macrolide antibiotics yield high concentrations in inflamed tissue, suggesting that their levels in gingival crevicular fluid (GCF) could be increased at gingivitis sites. However, the increased volume of GCF associated with gingivitis could potentially dilute macrolides. To determine whether these assumptions are correct, the bioavailability of systemically-administered azithromycin was compared in GCF from healthy and gingivitis sites.

Materials and methods: Experimental gingivitis was induced in one maxillary posterior sextant in nine healthy subjects. Contralateral healthy sextants served as controls. Subjects ingested 500 mg of azithromycin followed by a 250 mg dose 24 hours later. Four hours after the second dose, plaque was removed from experimental sites. GCF was collected from 8 surfaces in both the experimental and control sextants and pooled separately. GCF samples were subsequently collected on the 2nd, 3rd, 8th and 15th days and azithromycin content was determined by agar diffusion bioassay.

Results: On days 2 and 3, the pooled GCF volume at experimental sites was significantly higher than at control sites (P <0.01) and the total azithromycin mass in 30 second GCF samples pooled from experimental sites was significantly higher than at control sites (P < 0.02). However, there were no significant differences in azithromycin concentration between the experimental and control pools at any point. Concentrations exceeded 7.3 μg/ml on day 2 and 2.5 μg/ml on day 15.

Conclusions: Azithromycin concentrations are similar in GCF from gingivitis sites and healthy sites, suggesting that the processes that regulate GCF azithromycin concentration can compensate for local inflammatory changes.
INTRODUCTION

Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis are periodontitis-associated bacteria that are capable of invading cells in the soft tissue wall of periodontal pockets.\textsuperscript{1, 2} These invasive pathogens are difficult to eliminate by mechanical debridement alone,\textsuperscript{3} but adjunctive use of systemic antibiotics can enhance the therapeutic response to scaling and root planing (SRP).\textsuperscript{4, 5} As an example, azithromycin is a macrolide antibiotic that is active against \textit{A. actinomycetemcomitans}, \textit{P. gingivalis}, and other Gram negative anaerobes.\textsuperscript{6-8} Randomized clinical trials suggest that azithromycin enhances clinical attachment gain and pocket reduction in response to SRP in patients with aggressive and chronic forms of periodontitis.\textsuperscript{9-12} Adjunctive azithromycin also reduces the frequency of \textit{P. gingivalis} detection after SRP in patients with chronic periodontitis.\textsuperscript{12}

The pharmacokinetic properties of macrolides are influenced by their tendency to be taken up and released by many types of host cells. Macrolides accumulate at high levels inside phagocytes\textsuperscript{13, 14} and are actively transported by fibroblasts and oral epithelial cells.\textsuperscript{15, 16} The existence of these “macrolide reservoirs” may allow azithromycin and clarithromycin to attain high steady-state levels in gingiva.\textsuperscript{17, 18} It may also explain why steady-state concentrations of azithromycin are significantly higher and more sustained in gingival crevicular fluid (GCF) than in blood serum.\textsuperscript{19}

While previous studies have shown that the azithromycin content of periodontal tissues tends to increase in the presence of inflammation,\textsuperscript{17, 18} it is unclear whether inflammation is accompanied by increased macrolide levels in GCF. Although antibiotics are not used to treat gingivitis, experimental gingivitis is a well-characterized, reversible model of inflammation that exhibits characteristic features that can potentially influence drug levels in GCF. Gingivitis is associated with an increase in the volumetric density of fibroblasts and phagocytes in gingival connective tissue,\textsuperscript{20} increasing the local capacity for macrolide accumulation. Gingivitis is also accompanied by increased vascular and junctional epithelial permeability and a significant increase in the volume of interstitial fluid and GCF.\textsuperscript{26, 27} This could potentially elute macrolides from intracellular storage sites and, ultimately, dilute their levels in interstitial fluid and GCF. The purpose
of this pharmacokinetic study was to assess the influence of gingivitis on azithromycin distribution to the gingival crevice and its impact on therapeutic concentrations of azithromycin in GCF.

MATERIALS AND METHODS

Subject selection: Approval of this study was obtained from the Office of Responsible Research Practices at The Ohio State University. Healthy adult nonsmokers (3 males/6 females, mean age 30.4 years) in good systemic and periodontal health were recruited from the student population of the Ohio State University. Written informed consent was obtained from all subjects, using procedures approved by the Institutional Review Board. Subjects exhibited no evidence of clinical attachment loss, aside from localized minor facial gingival recession. Subjects with a history of drug allergy or recent medication use were excluded, as were pregnant females.

Study Design: Table 11 provides an overview of the timeline for this split-mouth prospective study. One week prior to a baseline periodontal examination, each subject was treated with a supragingival prophylaxis. A thermoplastic stent was fabricated to cover the maxillary posterior teeth on one randomly selected side. At the baseline examination, Gingival Index (GI)\textsuperscript{21} and Plaque Index (PI)\textsuperscript{22} were measured on the mesiobuccal and mesiolingual aspects of the maxillary premolars and molars. GCF samples were collected and pooled from the same eight posterior sites on each side as previously described.\textsuperscript{23} Briefly, sites were scaled to remove supragingival plaque, isolated with cotton rolls, and air dried to avoid contamination. GCF samples were then collected by positioning a paper strip (Periopaper\textsuperscript{TM}, Oraflow, Smithtown, NY) at the orifice of the sulcus for 30 seconds. GCF volume was measured with a calibrated gingival fluid measurement device (Periotron 6000, IDE Interstate, Amityville, NY). The eight samples from each side were pooled and stored at -20°C.

After the baseline examination, subjects were given a thermoplastic stent to facilitate induction of experimental gingivitis in one posterior region of the maxilla.\textsuperscript{24} Subjects were instructed to wear the stent during routine oral hygiene procedures to
prevent plaque removal from the experimental side, but were expected to maintain good oral hygiene at all other sites. Posterior sites in the contralateral quadrant served as healthy controls.

The subjects returned every seven days for the next three weeks for assessment of GI and PI at the control and experimental sites. At the three week visit, GCF samples were collected from the control sites. On the following day (Day 0), the subjects were administered an initial oral dose of 500 mg azithromycin followed by a 250 mg dose 24 hours later. Four to six hours later after the second dose, GI and PI were assessed at the control and experimental sites and plaque was gently removed from the experimental teeth. GCF samples were collected from the control and experimental sites, measured and pooled separately as previously described. A sample of peripheral blood was obtained by venipuncture. The subjects were instructed to immediately resume normal oral hygiene procedures. GI and PI assessments and GCF and blood samples were obtained in the same manner on days 2, 3, 8 and 15 after the initial dose of azithromycin.

To enhance objectivity and reduce delays that could lead to sample contamination or evaporation, GCF samples were collected by a team that included a clinical operator (NJ), a GCF measurement device operator (JW) and a data recorder (PL). The clinical operator assessed the PI and GI scores, isolated the GCF collection sites and collected GCF samples. The GCF measurement device operator measured the sample volumes and stored the samples in a cooled microtube. The GCF measurement device readout was not visible to the clinical operator during sample collection and the site of origin of the GCF sample was not visible to the GCF measurement device operator. Only the data recorder had direct access to this information.

**Sample preparation and analysis:** Prior to analysis, GCF was eluted from each pool of paper strips with 200 µl of liquid chromatography grade water, using a method previously described. The efficiency of elution, as assessed with [3H]-labeled macrolide (American Radiolabeled Chemicals, St. Louis, MO), was 67.4%. GCF eluates and blood serum samples (200 µl) were treated with 40 µl of 0.5 g/ml Na2CO3 and extracted three times with 1 ml diethyl ether. The extracts were dried under streaming nitrogen,
reconstituted in acetonitrile, and applied to sterile paper disks (BD Biosciences, Sparks, MD). After evaporation of acetonitrile, the azithromycin content of the disks was determined with an agar diffusion bioassay, using *Kocuria rhizophila* (ATCC 9341, American Type Culture Collection, Manassas, VA) as the indicator organism. The assay had a detection limit of 1 ng and an inter-assay coefficient of variability of <9% in calibrations with authentic azithromycin (US Pharmacoepeia, Rockville, MD). The reported azithromycin content of GCF incorporated a correction for the efficiency of elution.

**RESULTS**

At the baseline examination (22 days prior to the first dose of azithromycin), median PI and GI values at the control and experimental sites were 0 (Figure 12), and the mean volumes of pooled GCF samples from these sites were 0.98 µl and 0.88 µl, respectively (Figure 13). There were no significant differences between control and experimental sites.

During induction of experimental gingivitis, PI and GI values did not change at the control sites. At experimental sites, both indices increased to a median value of 1.5 within 21 days. With respect to PI and GI, the differences between control and experimental sites were significant at every observation point during this period (P ≤ 0.016, Wilcoxon signed-rank test). After initiation of the azithromycin regimen, PI and GI values decreased at experimental sites on day 1 (Figure 12). The decrease in PI was statistically significant (P = 0.019, Mann-Whitney rank sum test). GI decreased to baseline level within 7 days after plaque was removed from the experimental sites.

At control sites, there were no significant changes in pooled GCF volume during the period of gingivitis induction at experimental sites (Figure 13). In contrast, the GCF volume at experimental sites increased by more than two-fold. GCF volumes at experimental sites were significantly higher than control on days 1 through 3 after the initial azithromycin dose (P ≤ 0.01, paired t-test), although the volume at experimental sites decreased by approximately 40% between days 1 and 3. GCF volumes at control and experimental sites converged after day 3 and were not significantly different on days
8 and 15. Interestingly, there was a transient 30% decrease in mean control GCF volume between days 1 and 3. On day 15, however, the observed volumes were similar to those observed prior to administration of azithromycin.

The total azithromycin mass in 30 second GCF samples pooled from the experimental sites was consistently higher than control from day 1 to day 3 (Figure 14). The differences were significant on days 2 and 3 (P ≤ 0.02, paired t-test). Thereafter, the total azithromycin mass in experimental and control samples was not significantly different. Between day 1 and day 15, the total azithromycin mass in the experimental samples was closely correlated with GCF volume at the same sites, but was not significantly correlated with serum azithromycin concentration (Table 12). Over the same time interval, the total azithromycin mass in control samples was closely correlated with serum azithromycin concentration, but not with GCF volume.

On day 1, the azithromycin concentration in control GCF was higher than in experimental GCF (Figure 15). Thereafter, the concentrations at control and experimental sites converged and were nearly identical on days 8 and 15. While there were no significant concentration differences between control and experimental sites between day 1 and day 15, azithromycin levels in GCF was consistently higher than in blood (P < 0.011, repeated measures ANOVA, P < 0.05, Holm-Sidak test). The azithromycin concentration in control GCF was approximately 26-fold higher than in serum on day 1 and 560-fold higher on day 15. Between days 1 and 15, GCF azithromycin concentration at experimental sites did not exhibit a strong relationship with GCF volume or serum azithromycin concentration (Table 12). At control sites, GCF azithromycin concentration was not well correlated with GCF volume, but exhibited a significant correlation with serum azithromycin concentration.

**DISCUSSION**

The findings of this study demonstrate that azithromycin concentrations are not significantly different in GCF from gingivitis sites and healthy sites. Although the mass flow rate of azithromycin entering inflamed gingival crevices was almost twice that observed at healthy sites, the volume of GCF passing through crevices affected by
gingivitis was also twice that measured in healthy crevices. Azithromycin concentrations were 15 to 50-fold higher in GCF than in blood serum on days 1 and 2 and >50-fold higher at later time points. This suggests that the processes that regulate azithromycin concentration in GCF maintain a relatively narrow concentration range that is surprisingly independent of the degree of local inflammation.

Drug concentrations in GCF can potentially be influenced by several factors associated with gingivitis. Vasodilatation increases the amount of drug distributed to the vessels of the gingival plexus, from which GCF originates. Leakiness of vessels in the gingival plexus increases movement of drug from blood to interstitial fluid and also increases the volume of interstitial fluid. Increased permeability of the junctional epithelium allows an increased volume of interstitial fluid to enter the crevice. In the initial stage of experimental gingivitis, there is alteration of the junctional epithelium and gingival vessels exhibit vasculitis, loss of perivascular collagen, and increased neutrophilic migration out of the vessels. These are accompanied by increased vascular and junctional epithelial permeability. Drugs that enter the interstitial fluid seep through gingival connective tissue and eventually cross the junctional epithelium into the gingival crevice. However, a substantial amount of macrolide antibiotics may be taken up from interstitial fluid and concentrated inside fibroblasts, epithelial, inflammatory and immune cells. These cells account for a relatively large specific volume of gingiva in health and an even larger volume in the presence of gingivitis. Since these cells can serve as macrolide reservoirs, they are thought to enhance macrolide distribution to gingiva and account for the large concentration difference between blood serum and GCF.

These factors may also account for the differences observed between gingivitis and control sites with respect to the amount of azithromycin recovered from pooled 30 second GCF samples. At gingivitis sites, the amount of azithromycin recovered was initially larger than at control sites and changes over time were strongly correlated with changes in GCF volume. At control sites, which presumably exhibited normal vascular and junctional epithelial permeability, there was a lower azithromycin content in GCF and changes over time correlated with changes in serum azithromycin concentration. As gingivitis resolved, differences in vascular and junctional epithelial permeability
presumably disappeared and the azithromycin content of control and experimental GCF were very similar on days 8 and 15.

In this study, two doses of azithromycin (750 mg total) produced sustained and relatively high antimicrobial concentrations in GCF. Two weeks after the final dose, GCF azithromycin concentrations were above the minimal inhibitory concentrations for *A. actinomycetemcomitans* (0.25 - 2.0 µg/ml), *P. gingivalis* (0.125 - 1 µg/ml) and *Prevotella intermedia* (0.03 - 1 µg/ml).6-8

Azithromycin exhibits time-dependent killing activity. Its antimicrobial effects are more dependent on the amount of time it is in contact with bacteria than its concentration at the infection site. The efficacy of time-dependent antibiotics can be predicted by the amount of time that the antibiotic exceeds the MIC of the target pathogens.28 Assuming the observed azithromycin concentrations in GCF are representative of concentrations in gingival connective tissue, the results suggest that azithromycin could produce beneficial antimicrobial activity, even in patients who fail to complete the standard 1.5 g regimen.

Subjects were administered only two doses of azithromycin in this study because of concerns that azithromycin could reduce the severity of experimental gingivitis. Azithromycin reportedly penetrates and dissolves periodontal biofilm29 as well as pulmonary biofilm associated with diffuse panbranchiolitis.30 Moreover, azithromycin induces a transient decrease in GCF volume at healthy periodontal sites in parallel with transient reductions in the GCF content of IL-8, IL-1β and TNF-α.31 Some anti-inflammatory effects of azithromycin have been attributed to down-regulation of cytokine gene expression via nuclear factor-κB and activator protein-1.32 There is evidence that these were reasonable concerns. Azithromycin appeared to induce a decrease in PI and GI at experimental sites that was apparent on day 1 of the study. In addition, azithromycin induced a transient decrease in pooled GCF volume at control sites. This suggests that azithromycin may have also contributed to the rapid reduction of GCF volume observed at experimental sites after termination of experimental gingivitis.
CONCLUSIONS

The mass flow rate of azithromycin is higher at gingivitis sites than at healthy sites, but there is no significant difference in concentration. It is possible that elution of azithromycin from intracellular storage sites helps maintain this relatively narrow concentration range. Although the subjects were administered only half the standard total dose of azithromycin, its concentration in GCF remained above the minimal inhibitory concentration for several periodontal pathogens for at least two weeks after the final dose. These pharmacokinetic properties, in conjunction with its antimicrobial and anti-inflammatory activities, suggest that azithromycin is well suited for treating inflammatory periodontitis.

REFERENCES


<table>
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<th>Day</th>
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<tr>
<td>–29</td>
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<td>–22</td>
<td>Measure baseline PI and GI</td>
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<td>Collect baseline GCF samples</td>
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<td>Begin experimental gingivitis induction</td>
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<tr>
<td>–1</td>
<td>Measure PI and GI</td>
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<td>Collect control GCF sample</td>
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<tr>
<td>0</td>
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<tr>
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<td>Azithromycin dose 2</td>
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<tr>
<td>1 (+ 4 to 6 hours)</td>
<td>Remove plaque from experimental sites</td>
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<tr>
<td></td>
<td>Measure PI and GI</td>
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<td></td>
<td>Collect GCF and blood samples</td>
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<tr>
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<td>Participants resume oral hygiene</td>
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<tr>
<td>2, 3, 8, 15</td>
<td>Measure PI and GI</td>
</tr>
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<td></td>
<td>Collect GCF and blood samples</td>
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Table 11. Study timeline
Table 12. Correlations between azithromycin concentration, azithromycin mass flow rate and GCF volume at experimental and control sites

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
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<th>R Value, Control Sites</th>
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<td>Azithromycin in GCF (ng/30 seconds)</td>
<td>GCF volume</td>
<td>0.966*</td>
<td>0.597</td>
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</table>

[Azithromycin] = total azithromycin concentration.

* $P < 0.05$, ANOVA.
Figure 12. Changes in clinical indices observed during the study

Arrows indicate the beginning and end of the plaque accumulation during the experimental gingivitis phase. A: Plaque Index at control and experimental sites. Differences between the control and experimental sites were statistically significant at all time points from day –15 to day 1 (P < 0.05, Wilcoxon signed rank test). B: Gingival Index at control and experimental sites. Differences between control and experimental sites were statistically significant from day –15 to day 4 (P < 0.05).
Figure 13. Changes in pooled GCF volume at control and experimental sites

Each pool was derived from eight paper strip samples, with each strip collected for 30 seconds. The data are presented as mean + SEM. Arrows indicate the beginning and end of the experimental gingivitis phase. Asterisks denote significant differences between control and experimental volumes (P < 0.05, paired t-test).
Figure 14. Azithromycin content of GCF samples polled separately from experimental and control sites. Data are presented as mean ± SEM, and the arrow indicates the end of experimental gingivitis phase. Asterisks denote significant differences between control and experimental pools (P < 0.05, paired t-test).
Data are presented as mean + SEM. The arrow denotes the time plaque was removed at the end of the experimental gingivitis phase. Azithromycin concentrations in GCF from control and experimental sites were not significantly different. However, concentrations measured in control GCF were significantly higher than those found in blood at all observation points (P < 0.05, repeated measures ANOVA with post-hoc Holm-Sidak test).