IN VITRO RESISTANCE OF HUMAN PERIODONTAL ANAEROBIC BACTERIAL PATHOGENS TO TINIDAZOLE VERSUS METRONIDAZOLE.

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ABSTRACT

Objectives: Most bacterial species implicated as pathogens in human periodontitis are anaerobic in their metabolism. Systemic administration of metronidazole, an antibiotic specifically active against anaerobic bacteria, has been shown in multiple clinical trials to be beneficial in enhancing periodontal therapeutic outcomes beyond that attained by conventional mechanically-based forms of periodontal therapy alone, in large part by the drug inducing better reductions of major anaerobic pathogens in periodontal pockets. However, systemic metronidazole regimens in the treatment of periodontitis require multiple patient-administered drug doses per day, which may compromise treatment benefits in patients less compliant with prescribed oral drug consumption schedules. Tinidazole, a second-generation 2-methyl-5-nitroimidazole class antibiotic similar to metronidazole, also possesses marked antibacterial activity against anaerobic bacteria, and exhibits pharmacokinetic properties that enable its bioavailability with only a once-a-day oral drug dose, which may be an advantage for use in periodontitis patients unable to comply with more frequent drug dosing regimens. Little comparative data is available assessing the potential antimicrobial effects of tinidazole, as compared to metronidazole, against anaerobic periodontal pathogens, particularly “wild-type” clinical strains isolated from severely-diseased human periodontal pockets. As a result, this study tested fresh clinical subgingival isolates of selected anaerobic red and orange complex periodontal pathogens for their in vitro susceptibility to tinidazole, metronidazole, and three other antibiotics frequently employed in periodontal therapy.

Methods: Paper point subgingival plaque biofilm specimens were removed from 31 adults with severe periodontitis, and transported in VMGA III medium from various

United States private periodontal practices to the Oral Microbiology Testing Service Laboratory at Temple University School of Dentistry. Within 24 hours, the samples were serial diluted and plated onto enriched Brucella blood agar plates with either no antimicrobials added, or supplemented with either tinidazole at 16 mg/L, metronidazole at 16 mg/L, doxycycline at 4 mg/L, amoxicillin at 8 mg/L, or clindamycin at 4 mg/L, which represent recognized non-susceptible drug breakpoint concentrations for each of the antibiotics. After incubation at 37°C for 7 days in an 85% N_2-10% H_2-5% CO_2 anaerobic atmosphere, all plates were examined with established phenotypic criteria for selected anaerobic red and orange complex periodontal pathogens, including *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia/nigrescens*, *Parvimonas micra*, and *Fusobacterium nucleatum* group species. In vitro antibiotic resistance was noted when any of the test bacterial species displayed growth on one or more of the antibiotic-supplemented enriched Brucella blood agar plates. A paired t-test compared mean total subgingival proportions of the evaluated anaerobic red and orange complex periodontal pathogens per patient which were resistant in vitro to non-susceptible drug threshold concentrations of tinidazole as compared to metronidazole, as well as to doxycycline, amoxicillin, and clindamycin, with a *P*-value of ≤ 0.05 required for statistical significance.

Results: The study patients yielded an average 25.8% per patient of total subgingival proportions of the selected anaerobic red and orange complex periodontal pathogens. Among these species, *P. micra* was isolated from all (100%) study patients, and *P. intermedia/nigrescens* and *F. nucleatum* from 93.5% and 90.3% patients, respectively, with mean subgingival proportions of these species in positive patients

ranging from 1.8% to 9.7%. *T. forsythia* at mean subgingival levels of 1.8% was recovered from 54.8% of the patients, whereas subgingival *P. gingivalis* averaged 9.1% in 5 (16.1%) patients. Tinidazole and metronidazole at 16 mg/L threshold concentrations inhibited in vitro growth of all test periodontal pathogens, except for a tinidazole-resistant strain of *P. intermedia/nigrescens* in one patient that was additionally resistant in vitro to doxycycline, amoxicillin and clindamycin. No statistically significant differences were found between tinidazole and metronidazole in mean total subgingival proportions of anaerobic red and orange complex periodontal pathogens per patient exhibiting in vitro resistance to a 16 mg/L drug concentration ($P = 0.327$, paired t-test). However, significantly greater total subgingival proportions of anaerobic red and orange complex periodontal pathogens per patient were resistant in vitro to breakpoint concentrations of either doxycycline, amoxicillin, or clindamycin, as compared to tinidazole or metronidazole (all P-values < 0.006, paired t-test).

Conclusions: Tinidazole performed in vitro similar to metronidazole, but significantly better than doxycycline, amoxicillin, or clindamycin, in antimicrobial activity against freshly-isolated clinical strains of human subgingival anaerobic red and orange complex periodontal pathogens. As a result of its similar spectrum of antimicrobial inhibition against anaerobic bacteria, and its more convenient once-a-day oral drug dosing properties, tinidazole may be prescribed for clinical systemic use in place of metronidazole in severe human periodontitis treatment regimens where patient compliance with multiple dose per day systemic drug consumption is anticipated to be poor or difficult to attain.

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CHAPTER 1

INTRODUCTION

Human periodontitis is a bacterial-triggered, inflammatory-mediated, destructive form of periodontal disease, where progressive loss of gingival connective tissue attachment to teeth, and progressive loss of tooth-supporting alveolar bone, compromises the stability and function of teeth, and potentially leads to their loss from the oral cavity (Pihlstrom et al. 2005, Kinane et al. 2017).

Among the approximately 700 known microbial species and currently uncultivated phylotypes that inhabit the human oral cavity, only a subset are associated with the subgingival microbiome in periodontitis-affected patients (Colombo & Tanner 2019). Using multiple cluster and community ordination statistical analysis on microbiological data from 13, 261 subgingival plaque biofilm samples obtained from 185 patients exhibiting a wide range of clinically-healthy and diseased periodontal conditions, Socransky et al. (1998) designated as putative periodontal pathogens three bacterial species as members of a red complex associated with the most severe forms of clinical periodontal attachment loss, and 12 species in an orange complex associated with less severe forms of periodontal breakdown, while other evaluated microbial species were more associated with periodontal health or gingivitis.

Importantly, all three of the red complex periodontal pathogens (*Porphyromonas gingivalis, Tannerella forsythia,* and *Treponema denticola*), and most of the orange complex species (including *Prevotella intermedia, Prevotella nigrescens, Parvimonas micra,* and *Fusobacterium nucleatum* group species), are considered to be strictly

anaerobic in their metabolism (Rams & van Winkelhoff 2017), indicating a strong statistical association between certain anaerobic bacterial species and severity of human periodontitis.

Consistent with this, adjunctive administration of systemic metronidazole, an antibiotic specifically active against anaerobic bacteria, has been shown in multiple clinical trials to be beneficial in enhancing therapeutic outcomes in severe periodontitis patients beyond that attained by conventional mechanically-based forms of periodontal therapy alone (Loesche et al. 1996, 2005, Haffajee et al. 2007, Feres et al. 2012, Preus et al. 2013, Smiley et al. 2015), due in large part to the drug inducing better reductions of anaerobic periodontal pathogens in periodontal pockets (Loesche et al. 1984, Haffajee et al. 2008, Soares et al. 2014, Preus et al. 2015).

However, the need for patients to be compliant in taking systemic metronidazole doses as prescribed is critical to the drug's potential periodontal therapy value. Metronidazole is most often prescribed to be taken three times per day orally in 250-500 mg doses for 7-14 days (Slots 2004, Haffajee et al. 2007, Walters & Lai 2015). In a double-blind clinical study, only 56% of 18 study patients were considered adequately compliant with taking systemic metronidazole tablets three times per day as prescribed as an adjunct to non-surgical periodontal instrumentation and daily oral hygiene procedures (Loesche et al. 1993). Compliant patients experienced significantly better periodontal treatment outcomes than less compliant patients, with a mean post-treatment reduction of 8.3 teeth with periodontal surgical treatment needs among patients compliant with the systemic metronidazole dosing schedule, as compared to average reductions of 3.6 teeth

with periodontal surgery treatment needs in less compliant patients (Loesche et al. 1993). Thus, the need to take drug tablets three times per day was a complicating factor that markedly compromised the usefulness of metronidazole in periodontitis therapy.

These patient compliance problems may be overcome with administration of tinidazole, which may serve as a potential alternative to metronidazole in combatting anaerobic periodontal pathogens in periodontitis lesions (Granizo et al. 2009). Tinidazole is a second-generation 2-methyl-5-nitroimidazole class antibiotic structurally similar to metronidazole (Figure 1) which has received relatively little research attention for its potential use against periodontal infections (Manso et al. 2008).

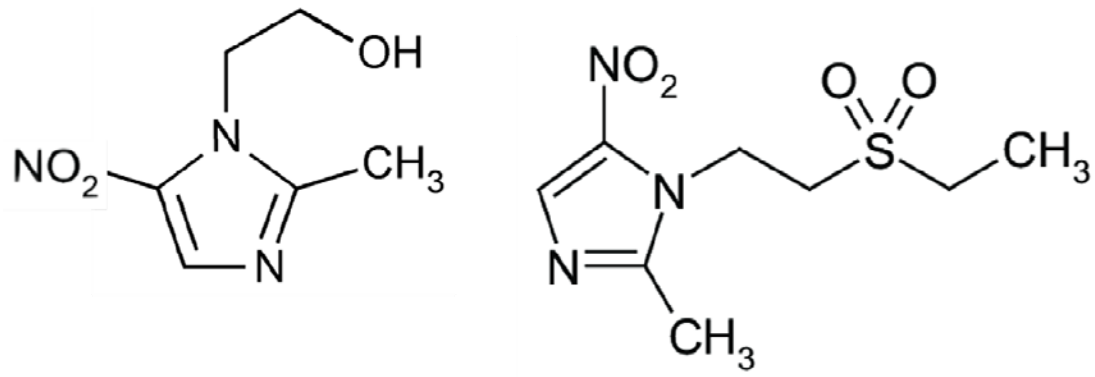

Figure 1. Molecular structures of metronidazole (left) and tinidazole (right), adapted from Castillo et al. (2010).

Tinidazole exerts marked antimicrobial activity against anaerobic bacteria and protozoa similar to or greater than metronidazole (Nord 1982, Rao & Shivananda 2000, Petrina et al. 2017, Pandey et al. 2018), and has pharmacokinetic properties that enable its bioavailability in serum, gingival crevicular fluid, and within gingival tissues at therapeutic levels with only a once-a-day oral drug dose (Liew et al. 1991), which offers

an advantage in comparison to metronidazole relative to attaining patient consumption compliance. Tinidazole has a 12-14 hour serum half-life, which is approximately double that found with metronidazole (von Konow & Nord 1982, Wood et al. 1982), which permits less frequent drug dosing. Following a single two-gram oral drug dose to 10 adults with moderate to severe periodontitis, mean gingival crevicular fluid concentrations of 13 mg/L of tinidazole were detected after 24 hours (Liew et al. 1991), indicative of the potential sustained antimicrobial effect that tinidazole may mount against anaerobic periodontal pathogens in subgingival sites.

Research is presently rare and of limited scope on the use of tinidazole in the treatment of periodontal diseases. Systemic tinidazole has shown efficacy in treatment of periodontitis in various breeds of dogs, where significantly greater probing depth reductions were found with when tinidazole was administered in addition to tooth scaling, as compared to scaling alone, leading to complete elimination of *P. gingivalis*-like subgingival isolates in the dogs (Sarkiala et al. 1993). In humans, systemic tinidazole prescribed as an adjunct to non-surgical periodontal instrumentation on periodontitis patients has been reported to provide a significantly higher "effective rate" than systemic metronidazole (73.1% versus 43.5%) (Wang et al. 1996), and induced significantly greater favorable changes in probing depth, clinical periodontal attachment level, and gingival inflammation in smoking periodontitis patients at six weeks post-treatment, as compared to conventional root scaling alone (Kiany et al. 2016).

Also limited are data on the antimicrobial effects of tinidazole on anaerobic red and orange complex periodontal pathogens. Minimal inhibitory concentrations of

tinidazole for 90% of organisms evaluated (MIC_{90} values) have been reported to be 4 mg/L for *P. intermedia* (among 10 periodontal isolates tested), and 1 mg/L for *F. nucleatum* (10 isolates), with only one isolate of *P. intermedia* revealing in vitro resistance to tinidazole with MIC values of 16 mg/L (Alou et al. 2009). Another study found in vitro antimicrobial synergism of tinidazole with either clindamycin, amoxicillin/clavulanic acid, or levofloxacin against a constructed mixed bacterial inoculum of periodontal origin, including *F. nucleatum*, *P. buccae*, *Veillonella* species, *Capnocytophaga* species, and *Streptococcus* species (Alou et al. 2010).

No comparative data is available for a wider range of anaerobic periodontal pathogens, particularly wild-type clinical strains isolated from severely-diseased human periodontal pockets, which assess the potential antimicrobial effects of tinidazole as compared to metronidazole. As a result, the objective of this study was to test fresh clinical subgingival isolates of selected anaerobic red and orange complex periodontal pathogens for their in vitro susceptibility to tinidazole, metronidazole, and three other antibiotics frequently employed in periodontal therapy, and to compare the in vitro anti-periodontal anaerobe activity of tinidazole to metronidazole and the other three antibiotics.

CHAPTER 2

MATERIALS AND METHODS

Laboratory Facilities

All procedures in this study were performed using the facilities of the Oral Microbiology Testing Service (OMTS) Laboratory located at the Temple University Maurice H. Kornberg School of Dentistry on the Temple University Health Sciences Center campus in Philadelphia, Pennsylvania. Since the present study was non-clinical and laboratory-based, with study data obtained through secondary use of subgingival plaque biofilm samples without any intervention or interaction with living individuals, and not involving any identifiable private information, the research activity did not involve human subjects, as defined by United States Department of Health and Human Services regulations at 45 CFR part 46.116(f), and did not require a human subjects institutional review board approval, per a written determination issued by the Temple University Human Subjects Protections Institutional Review Board.

Subgingival Plaque Biofilm Specimens

Subgingival plaque biofilm specimens were utilized from 31 adults with severe periodontitis from whom subgingival samples were submitted to the OMTS Laboratory for microbiological analysis and antibiotic resistance testing by subscribing private practicing periodontists extramural to Temple University. The subgingival specimens were normally discarded by the OMTS Laboratory after completion of the requested microbiological testing, but were additionally used in this study after removal of all unique patient identifiers. As a result, other than knowledge that the patients were 35

years of age or older, and identified by their treating periodontist as having severe periodontitis untreated at the time of the microbiological sampling, no additional information is available for the patients, such as their exact age, gender, systemic health history, medications, smoking status, or nature of their clinical/radiographic periodontal parameters beyond their overall periodontal diagnosis.

The subgingival specimens were obtained by the treating periodontists, as instructed by the OMTS Laboratory, by removing supragingival plaque on the patients from 3-5 periodontal sites exhibiting moderate (5-6 mm) to deep periodontal probing depths (≥ 7 mm) and gingival inflammation, and isolating them with cotton rolls and air drying to avoid saliva contamination in the microbial samples. Following these steps, one to two sterile paper points were then advanced with sterile forceps into each isolated periodontal site for approximately 10 seconds in order to collect subgingival plaque biofilm specimens for microbial culture. The paper points were then placed together into a single glass vial containing 6-8 glass beads of 3 mm in diameter, and 2.0 ml of pre-reduced, anaerobically sterilized and stored Möller's VMGA III transport media (Möller 1966), which possesses a high preservation capability for oral microorganisms after sampling during transit to the laboratory (Möller 1966, Dahlén et al. 1989, Dahlén et al. 1993).

The pooled subgingival plaque biofilm samples were delivered within 24 hours to the OMTS Laboratory in Philadelphia, Pennsylvania, which has been in continuous operation since its founding in 1991. The OMTS Laboratory is state-licensed for high-complexity bacteriological analysis by the Pennsylvania Department of Health (Clinical

Laboratory Permit No. 021872) as an oral microbiology reference laboratory. The OMTS Laboratory is also federally certified by the US Department of Health and Human Services to be in compliance with Clinical Laboratory Improvement Amendments (CLIA) regulations (CLIA Certificate No. 39D0707385), and as a result, adheres to all of the proficiency testing, quality control, patient test management, personnel requirements, and quality assurance standards required of clinical laboratories engaged in diagnostic testing of human specimens in the United States (Rauch & Nichols, 2007).

Microbial Culture and In Vitro Antibiotic Resistance Testing

Upon arrival at the OMTS Laboratory, the VMGA III vials were warmed to 37ºC for 10 minutes prior to processing in order to liquefy gelatin in the VMGA III transport medium. The sampled plaque organisms were then mechanically dispersed in the medium with a Vortex mixer at the maximal setting for 45 seconds. Serial 10-fold dilutions of the dispersed bacteria were carried out in Möller's VMG I anaerobic dispersion solution, comprised of pre-reduced, anaerobically sterilized 0.25% tryptose-0.25% thiotone E peptone-0.5% NaCl (Möller 1966). Using a sterile bent glass rod, 0.1 ml aliquots of appropriate dilutions were plated onto pre-reduced, enriched Brucella blood agar (EBBA), comprised of 4.3% Brucella agar (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin, and 0.00005% menadione. The EBBA inoculated plates were incubated at 37°C for 7 days in a upright heated incubator (Caron, Marietta, OH, USA) in jars containing an 85% N_2-10% H_2-5% CO_2 anaerobic atmosphere introduced by an Anoxomat™ Mark II automatic jar

evacuation-replacement system (Advanced Instruments, Inc., Norwood, MA, USA) (Brazier & Smith 1989), and used to determine presence and levels of total anaerobic viable counts, and the following anaerobic red and orange complex periodontal pathogens: *Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia/ nigrescens, Parvimonas micra,* and *Fusobacterium nucleatum* group species.

Additional 0.1 mL aliquots of subgingival sample dilutions were inoculated onto EBBA primary isolation plates supplemented with either tinidazole at 16 mg/L, metronidazole at 16 mg/L, doxycycline at 4 mg/L, amoxicillin at 8 mg/L, or clindamycin at 4 mg/L (all antibiotics obtained as pure powder from Sigma-Aldrich, St. Louis, MO, USA), and incubated anaerobically for 7 days. These antimicrobial concentrations represent non-susceptible/resistant breakpoint concentrations against anaerobic bacteria for amoxicillin, clindamycin, and metronidazole as recommended by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2012), and for doxycycline as recommended by the French Society for Microbiology (French Society of Microbiology Antibiogram Committee 2010). Since breakpoint concentrations for tinidazole are not established, and are viewed to be equivalent to those applicable to metronidazole (Alou et al. 2010), the 16 mg/L non-susceptible/resistant breakpoint concentration against anaerobic bacteria used for metronidazole was also applied to tinidazole in this study. In vitro resistance to the antibiotic breakpoint concentrations was recorded when test species growth was noted on the respective antibiotic-supplemented EBBA plates (Slots et al. 1988, Feres et al. 1999, Rams et al. 2011, Rams et al. 2014). *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridium*

perfringens ATCC 13124, and a multi-antibiotic-resistant clinical periodontal isolate of *Fusobacterium nucleatum* were used as positive and negative quality controls for all antibiotic resistance testing on drug-supplemented EBBA plates.

Identification of Test Bacterial Species

On anaerobically incubated EBBA, *P. gingivalis* identification was based on colony morphology and brown-black pigmentation, lack of autofluorescence with long-wave ultraviolet light (Slots & Reynolds 1982), and a positive CAAM test for trypsin-like activity (Slots 1987). *T. forsythia* isolates were identified as gram-negative, non-motile, anaerobic rods exhibiting grey-pink speckled, convex, pinpoint colonies seen with a stereomicroscope, lack of long-wave ultraviolet light autofluorescence, and positive for trypsin-like enzyme activity (Rams & van Winkelhoff 2005). *P. intermedia/nigrescens* was recognized as autofluorescent red-positive, black-pigmented colonies exhibiting lactose MUG-test negative (Alcoforado et al. 1987) and trypsin CAAM test-negative reactions. *P. micra* was identified as small (minute to 1.0 mm in diameter), shiny, non-hemolytic, mainly opaque white, circular, convex surface colonies on anaerobically incubated EBBA (Rams et al. 1992). *F. nucleatum* isolates were presumptively identified, as specified by Jousimies-Somer et al. (2002), as gram-negative, non-motile, anaerobic, slender, fusiform rods with pointed cell ends (needle-shaped morphology), exhibiting circular, entire, raised, catalase-negative, non-pigmented, non-agar pitting, "bread crumb"-like or speckled colonies under a magnification loupe on anaerobically incubated EBBA, and demonstrating an autofluorescent chartreuse (pale yellow-green) colony color when exposed in a dark room to long-wave ultraviolet light (Brazier 1986).

Figure 2 provides an overall summary of the various culture media onto which the subgingival plaque biofilms were plated for incubation and recovery of selected anaerobic red and orange complex periodontal pathogens.

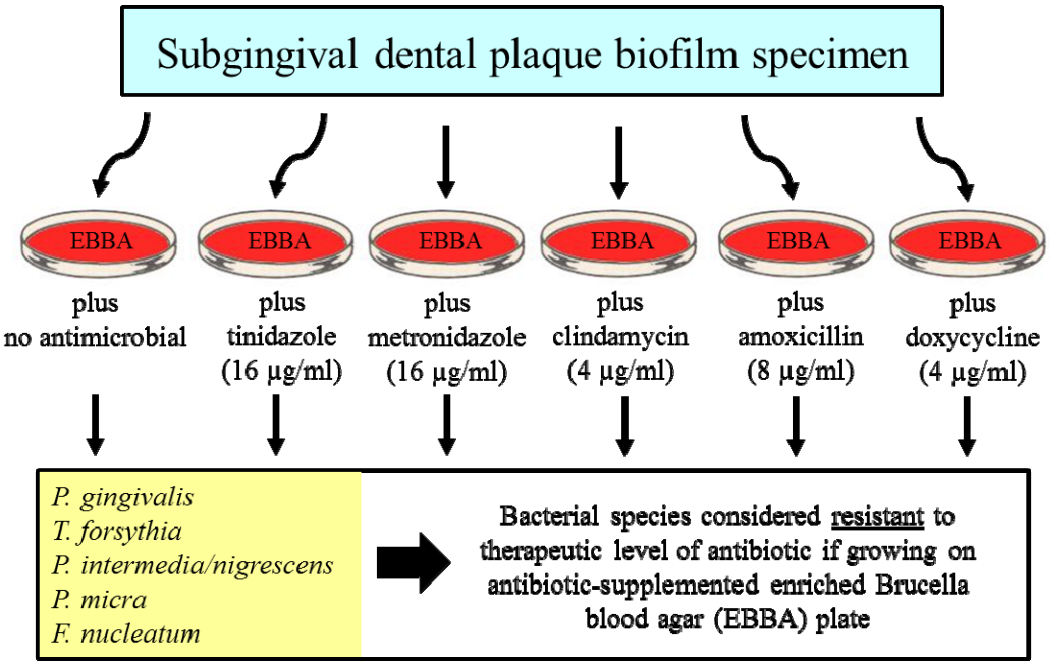

Figure 2. Flow chart for processing of human subgingival plaque biofilm specimens.

Proportional subject recovery of the above test bacterial species was calculated as the percent recovery of each test species colony forming units (CFU) among the total cultivable subgingival anaerobic viable count as determined on non-antibiotic supplemented EBBA plates.

All culture media preparation and specimen inoculation were carried out in a standardized fashion, and all microbiological analysis were performed on a blind basis by a single oral microbiology laboratory technician (Jackie Sautter), with subsequent review

by the laboratory director (Dr. Thomas E. Rams), in the OMTS Laboratory without knowledge of the diagnosis or clinical status of the study subjects.

Data Analysis

For each of the test bacterial species, the number and proportion of organism-positive patients from non-antibiotic containing EBBA plates was determined, along with the organism's mean subgingival proportional recovery and standard deviation, as well as the number and proportion of patients positive for resistant strains of the species on the various antibiotic-supplemented EBBA plates. Total subgingival proportions of the evaluated anaerobic red and orange complex periodontal pathogens (Carvalho et al. 2005, Page & Rams 2013, McCawley et al. 2018) were determined by summing together individual species data for each patient, and then calculating total mean values across all patients. A paired t-test compared mean total subgingival proportions of the evaluated anaerobic red and orange complex periodontal pathogens per patient which were resistant in vitro to non-susceptible drug threshold concentrations of tinidazole as compared to metronidazole, as well as to doxycycline, amoxicillin, and clindamycin, with a *P*-value of ≤ 0.05 required for statistical significance. The PC-based STATA/SE 14.2 for Windows (StataCorp PL, College Station, TX, USA) 64-bit statistical software package was used in the data analysis.

CHAPTER 3

RESULTS

Subgingival Plaque Biofilm Species

Table 1 lists the distribution of recovered test subgingival bacterial species in the 31 untreated severe periodontitis study subjects.

Table 1. Presence and Proportional Subgingival Recovery of Selected Anaerobic Periodontal Pathogens in 31 Adults with Severe Periodontitis

Organism	No. (%) of positive patients	% recovery in organism-positive patients ± SD	Range %
Red complex species:			
P. gingivalis	5 (16.1)	9.1 ± 7.4	1.0-21.0
T. forsythia	17 (54.8)	1.8 ± 1.4	0.4-5.9
Orange complex species:			
P. intermedia/nigrescens	29 (93.5)	9.7 ± 12.9	0.1-46.7
P. micra	31 (100)	6.6 ± 3.9	0.7-15.0
F. nucleatum	28 (90.3)	8.4 ± 5.4	1.2-20.4

P. micra was isolated from all (100%) study patients, and *P. intermedia/ nigrescens* and *F. nucleatum* from 93.5% and 90.3% patients, respectively, with mean subgingival proportions of these species in positive patients ranging from 1.8% to 9.7%.

T. forsythia at mean subgingival levels of 1.8% was recovered from 54.8% of the patients, whereas subgingival *P. gingivalis* averaged 9.1% in 5 (16.1%) patients.

Overall, the study patients yielded an average 25.8% per patient of total subgingival proportions of the selected anaerobic red and orange complex periodontal pathogens.

In Vitro Antibiotic Resistance Among Test Bacterial Species

Table 2 lists among test bacterial species the distribution of in vitro resistance to non-susceptible antibiotic breakpoint concentrations.

Table 2. In Vitro Resistance of Selected Anaerobic Periodontal Pathogens to Non-Susceptible Antibiotic Breakpoint Concentrations

Organism (No. of positive patients)	TIN 16 mg/L[a]	MET 16 mg/L	AMOX 8 mg/L	CLIND 4 mg/L	DOX 4 mg/L
Red complex species:					
P. gingivalis (5)	0[b]	0	1 (20.0)	2 (40.0)	0
T. forsythia (17)	0	0	4 (23.5)	11 (64.7)	1 (5.9)
Orange complex species:					
P. intermedia/nigrescens (29)	1 (3.4)	0	16 (55.2)	15 (51.7)	11 (37.9)
P. micra (31)	0	0	0	20 (64.5)	9 (29.0)
F. nucleatum (28)	0	0	0	0	0

[a] non-susceptible breakpoint concentration of antibiotic used in vitro.

[b] No. (%) of organism-positive patients demonstrating in vitro resistance of

organism to non-susceptible breakpoint concentration of antibiotic.

Key to table: TIN = tinidazole, MET = metronidazole, AMOX = amoxicillin, CLIND = clindamycin, DOX = doxycycline

Tinidazole and metronidazole at 16 mg/L breakpoint threshold concentrations inhibited in vitro growth of all test periodontal pathogens, except for a tinidazole-resistant strain of *P. intermedia/nigrescens* in one patient that was additionally resistant in vitro to doxycycline, amoxicillin and clindamycin. Figure 3 provides a representative view of the in vitro antimicrobial activity of tinidazole versus metronidazole.

Figure 3. EBBA culture plates of subgingival isolates from a study patient with severe periodontitis. *P. intermedia/nigrescens* (black colonies) comprised 46.3%, *P. micra* (small bright white colonies) 12.2%, and *F. nucleatum* 9.8%, of cultivable isolates on EBBA medium without any antimicrobial agents (left). These species were absent on EBBA plates supplemented with tinidazole (center) or metronidazole (right), where only periodontal health-associated viridans streptococci group species were recovered.

Total subgingival proportions per patient of anaerobic red and orange complex periodontal pathogens resistant to tinidazole averaged 0.2 ± 0.8 (SD) %; to metronidazole

0%; to doxycycline 6.4 ± 11.6 (SD) %; to amoxicillin 5.6 ± 9.7 (SD) %; and to clindamycin 9.5 ± 12.0 (SD) % (Figure 4).

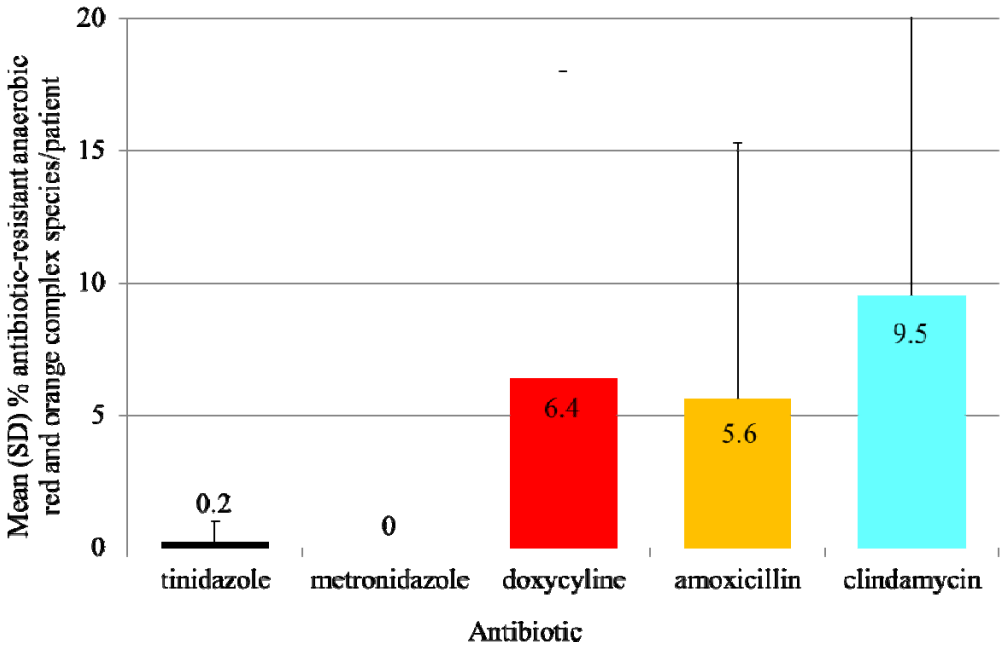

Figure 4. Average total subgingival proportions per patient of anaerobic red and orange complex periodontal pathogens resistant in vitro to test antibiotics.

No statistically significant differences were found between tinidazole and metronidazole in mean total subgingival proportions of anaerobic red and orange complex periodontal pathogens per patient exhibiting in vitro resistance to a 16 mg/L drug concentration ($P = 0.327$, paired t-test). However, significantly greater total subgingival proportions of anaerobic red and orange complex periodontal pathogens per patient were resistant in vitro to breakpoint concentrations of either doxycycline, amoxicillin, or clindamycin, as compared to tinidazole or metronidazole (all P-values < 0.006, paired t-test).

CHAPTER 4

DISCUSSION

The major findings from this study are that tinidazole performed similarly to metronidazole with regard to in vitro inhibition of major anaerobic red and orange complex periodontal pathogens that were freshly cultivated from severe periodontitis patients. No statistically significant differences were found between tinidazole and metronidazole in mean total subgingival proportions of anaerobic red and orange complex periodontal pathogens per patient exhibiting in vitro resistance to a 16 mg/L drug concentration of either antibiotic. Only one of 29 (3.4%) fresh subgingival isolates of *P. intermedia/nigrescens* exhibited in vitro resistance to 16 mg/L of tinidazole (Table 2), similar in extent to the one of 10 clinical periodontal isolates of *P. intermedia* reported by Alou et al. (2009) to be resistant in vitro to 16 mg/L of tinidazole.

The present study nearly triples available data on the number of *P. intermedia/ nigrescens* and *F. nucleatum* clinical periodontal isolates tested for tinidazole resistance, and provides for the first time evaluation of *P. gingivalis*, *T. forsythia*, and *P. micra* subgingival strains. It is noteworthy that all of the evaluated anaerobic red and orange complex periodontal pathogens, expect for a single strain of *P. intermedia/ nigrescens*, were susceptible to therapeutic concentrations of tinidazole that are clinically-attainable in periodontal pockets.

An additional important finding from the present study is the significantly better in vitro inhibition that tinidazole displayed against anaerobic periodontal pathogens as compared to that attained by either doxycycline, amoxicillin, or clindamycin (Figure 4).

Significantly greater total subgingival proportions of the evaluated anaerobic red and orange complex periodontal pathogens per patient were resistant in vitro to breakpoint concentrations of either doxycycline, amoxicillin, or clindamycin, as compared to tinidazole. With increasing concerns about the development over time of greater levels of antibiotic resistance among periodontal bacterial pathogens (Rams et al. 2014), tinidazole may prove to be a valuable alternative to antibiotics traditionally employed in periodontal therapy regimens as a means to help minimize spread of antibiotic resistance in populations as a result of dental care. For example, whereas recent data has uncovered marked increases over a 10-year period in periodontal *P. micra* in vitro resistance to clindamycin and amoxicillin (Rams et al. 2018), no similar in vitro resistance to tinidazole was found among the 31 fresh *P. micra* subgingival isolates evaluated in the present study which were all inhibited by 16 mg/L of tinidazole (Table 2).

It is important to note that tinidazole has an antimicrobial spectrum specific to anaerobic bacteria, with only limited antimicrobial effects against non-anaerobic bacteria (Nord 1982, Rao & Shivananda 2000, Petrina et al. 2017, Pandey et al. 2018). This has been attributed to intracellular reduction of the drug in the presence of a low oxidation-reduction potential and a ferredoxin system associated with anaerobic bacteria, but not aerobic microorganisms (Nord 1982, Manso et al. 2008). Thus, in clinical circumstances where a severe periodontitis patient presents with a mixture of both strictly anaerobic and facultatively anaerobic (such as *Aggregatibacter actinomycetemcomitans*) and/or aerobic periodontal pathogens (such as *Streptococcus constellatus*), combined use of tinidazole with another antibiotic, such as amoxicillin, may be indicated to broaden the

antimicrobial spectrum of drug chemotherapy. Combined antimicrobial spectrums of tinidazole with amoxicillin, or other appropriate antibiotics, will likely inhibit a wider array of periodontal pathogens than individual antibiotics, and reduce the risk of encountering periodontal pathogens resistant to both drugs (Rams et al. 2014). Added to this benefit is the reported antimicrobial synergism exhibited in vitro by tinidazole in combination with either clindamycin, amoxicillin/clavulanic acid, or levofloxacin (Alou et al. 2010). As pointed out by Manso et al. (2008), additional pharmacokinetic studies are needed to determine the best dosing schedules for such combined antibiotic regimens in light of differing half-life values for tinidazole and other antibiotics, such as amoxicillin.

Limitations of the present study need to be appreciated. No detailed patient data was available to examine potential gender- or age-specific differences in periodontal pathogen in vitro antibiotic resistance. Only selected anaerobic red and orange complex periodontal pathogens were detected in the subgingival plaque biofilm specimens evaluated, without assessment of additional periodontal pathogens which may possess more diverse and differing in vitro antibiotic resistance profiles. Only phenotypic criteria were employed to identify the targeted anaerobic red and orange complex periodontal pathogens, instead of potentially more precise molecular or biochemical methods. However, recent validation studies confirmed that the phenotypic methods used in the present study to identify *P. gingivalis* and *P. intermedia/nigrescens* clinical isolates highly correlate to species identification obtained via proteomic spectral fingerprinting of bacterial ribosomal protein profiles by matrix-assisted laser desorption/ionization time-

of-flight mass spectrometry (Rams et al. 2016, 2018). This reduces the possibility of misclassification of evaluated microbial species with the employed phenotypic identification methodology. Further, exact minimal inhibitory concentration values of the test antibiotics against the detected anaerobic red and orange complex periodontal pathogens were not determined with the laboratory methods followed, and antibiotic-resistance genes in the test periodontal pathogens were not studied.

Potential clinical application of tinidazole in periodontal therapy additionally requires clinical consideration of possible adverse drug side-effects and interactions with other patient medications. According to the drug package insert for Tindamax® (tinidazole) tablets (Mission Pharmaceutical Company, San Antonio, TX, USA) (available at https://www.accessdata.fda.gov/drugsatfda_docs/label/2007/021618s003lbl.pdf), the most common adverse side effects of multiple-day doses of tinidazole are a metallic/bitter taste (6.3% of patients), nausea (4.5% of patients), anorexia (2.5% of patients), dyspepsia/cramps/epigastric discomfort (1.4% of patients), vomiting (0.9% of patients), constipation (1.4% of patients), weakness/fatigue/malaise (1.1% of patients), dizziness (0.5% of patients), and headache (0.7% of patients). More rarely, broncho-spasm, dyspnea, coma, confusion, depression, furry tongue, pharyngitis and reversible thrombocytopenia have been associated with tinidazole use. Interactions between tinidazole and other patient medications are thought to be the same as those encountered with metronidazole (Miljkovic et al. 2014), which primarily involve potentiation of coumarin anticoagulants, lithium, intravenous phenytoin, cyclosporine, tacrolimus, and fluorouracil. An increased tinidazole elimination rate is associated with CYP3A4

inducing drugs, and increased tinidazole retention with CYP3A4 inhibiting medications. Tinidazole is contraindicated in pregnant patients in the first trimester, nursing mothers unless breast-feeding is stopped during drug use and for 3 days following the last dose, in persons who have taken disulfiram within the last two weeks, and in individuals consuming alcohol within a 3-day period.

Finally, the drug package insert for Tindamax® (tinidazole) tablets (available at https://www. accessdata.fda.gov/drugsatfda_docs/label/2007/021618s003lbl.pdf) also contains a United States Food and Drug Administration-mandated black-box warning of a potential cancer risk with tinidazole use, which is based on findings of cancer in various animal models with metronidazole. At present, no studies have been reported which specifically link tinidazole use to any type of human cancer. Studies on the risk of human cancer from 5-nitroimidazole class antibiotics as a group, which encompass both tinidazole and metronidazole, are presently inclusive and do not mirror findings in animal models (Friedman et al. 2009, Adil et al. 2018).

CHAPTER 5

CONCLUSIONS

Tinidazole performed in vitro similar to metronidazole, but significantly better than doxycycline, amoxicillin, or clindamycin, in antimicrobial activity against freshly-isolated clinical strains of human subgingival anaerobic red and orange complex periodontal bacterial pathogens.

As a result of its similar spectrum of antimicrobial inhibition against anaerobic bacteria, as evidenced by the present in vitro study findings, and its more convenient once-a-day oral drug dosing properties, tinidazole may be considered and prescribed for clinical systemic use in place of metronidazole in severe human periodontitis treatment regimens where patient compliance with multiple dose per day systemic drug consumption is anticipated to be poor or difficult to attain.

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