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Relative Efficacy of Innovative Endodontic Antimicrobial Agents

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RELATIVE EFFICACY OF INNOVATIVE ENDODONTIC ANTIMICROBIAL AGENTS

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ABSTRACT

Purpose: Novel endodontic antimicrobial agents would potentially improve outcomes of endodontic treatment, including regenerative endodontic therapy. The aim of this study was to determine the efficacy of a novel experimental compound, analogue #66, compared to tigecycline and other controls in an ex-vivo model.

Materials and Methods: MIC determination with tigecycline and analogue #66 was done for *Streptococcus mutans* and *Streptococcus intermedius*. Forty single rooted extracted teeth were inoculated weekly for three weeks with *S. intermedius* to create a biofilm. All teeth were instrumented to 40/06 to working length and irrigated with 5% NaOCl. Teeth were randomly divided into 4 groups (n=10 per group): (1) DMSO as negative control, (2) calcium hydroxide as positive control, (3) analogue #66 (10 mg/mL) group and (4) tigecycline (10 mg/mL).
Microbiological sampling of all groups was performed after one week.

Results: Log colony forming units (CFUs) from calcium hydroxide (5.07 $\pm$ 0.12), analogue #66 (4.94 $\pm$ 0.15) and tigecycline (0.58 $\pm$ 1.22) showed statistically significant ($P < 0.05$) bacterial reduction when compared with negative control (5.71 $\pm$ 0.17) ($P<0.05$). Although higher microbial reduction was observed in analogue #66 compared with calcium hydroxide, the difference was not statistically significant ($P >0.05$). Residual CFUs were lowest in the tigecycline group and were significantly lower than all other groups ($P <0.05$).

Conclusion: Calcium hydroxide, analogue #66 and tigecycline all showed significant antimicrobial efficacy against *S. intermedius* biofilm. Tigecycline was the most efficacious in this model.

Keywords: Substance #66; tigecycline, antimicrobial; intracanal medicaments; regenerative endodontics;

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CFU	Colony forming units
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
hDHFR	Human dihydrofolate reductase
MIC	Minimal inhibitory concentration
NaOCl	Sodium hypochlorite
RET	Regenerative endodontic therapy
*Sm*DHFR	*S. mutants* dihydrofolate reductase
TAP	Triple antibiotic paste
TMQ	Trimetrexate
UAB	University of Alabama at Birmingham

INTRODUCTION

Success in endodontic treatment depends on the degree of prevention or elimination of bacterial infections in the root canal environment (1). Intracanal medicaments and irrigation solutions are an important step to enhance root canal disinfection. Currently, intracanal medication is often used after chemo-mechanical preparation, in cases with infection, to improve disinfection (2). Interappointment medicaments can be used for any non-surgical endodontic treatment.

Regenerative endodontic therapy (RET) was introduced in the last two decades and is now being performed even in selected cases with mature apex (3). The effectiveness of RET depends on effective infection control and adequate disinfection of the root canal system (4). Inadequate disinfection of roots leading to bacterial persistence in root canals can significantly interfere with root maturation and healing (5). The current disinfection protocol includes dressing the canals with medicaments after irrigating with sodium hypochlorite (NaOCl) (6). Two commonly used medicaments are calcium hydroxide or triple antibiotic paste (TAP) (ciprofloxacin, minocycline, and metronidazole) (7). However, both medicaments have their downfalls. Calcium hydroxide's effectiveness is unclear, as some bacteria are resistant, and its clinical efficacy has not been definitively demonstrated (8) In addition to having several resistant gram-positive bacteria, calcium hydroxide can also induce the state of viable but uncultivable state such that after its removal, the bacteria can continue to grow (9). TAP has been shown in clinical outcome studies to be more effective than calcium hydroxide, (10), (11) but has the significant side effect of

crown discoloration. Non-specific antimicrobial biocides currently used can potentially cause changes in the remaining dentin which may interfere and inhibit potentials for regeneration (12). Moreover, non-specific antimicrobials suppress all classes of bacteria, this may allow for virulent organisms to repopulate the pulp space.

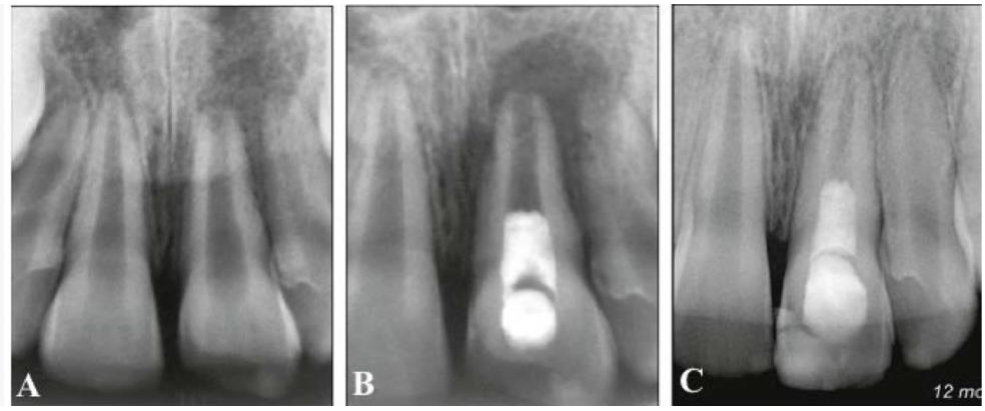


Figure 1. A series of periapical radiographs of regeneration procedure completed on tooth #9. (A). Initial radiograph. (B). Radiograph taken right after 1 month. (C). twelve months after initial Treatment. (13).

The most effective endodontic disinfection agent available is NaOCl (2.5-6%) (14). However, regarding RET, concerns have been raised that this agent may interfere with the ability of stem cells to attach to dentin, (15). In addition, NaOCl is not stable enough to have a residual effect in the root canal system to maintain an aseptic environment for regeneration to occur. For these reasons, although NaOCl is a very effective disinfection irrigant, it cannot be used as a medicament.

Folate is essential for all living organisms and is a key component in the production of cofactors required to synthesis genetic materials. Dihydrofolate reductase (DHFR) is the enzyme involved in the production of these cofactors (16). Inhibition of DHFR leads to depletion of intracellular folate, which in turn limits cellular growth. Currently, antifolate drugs on the market are used to treat bacterial infections, parasitic infections,

and cancer chemotherapy (17). In this previous publication, DHFR inhibitors targeting cariogenic bacteria were first explored. *S.mutans'* DHFR inhibitor sequence shares only 28% similarity with the human DHFR (hDHFR) sequence and was selected as a target in that study to design a new antimicrobial drug and was designated as Analogue #66.

Analogue #66 was specifically designed to target against bacterial DHFR (*Sm*DHFR) without interfering with human DHFR (hDHFR). Analogue #66 was shown to be effective at reducing bacterial growth of *S. mutans* at 6.25 μM: *S. sanguinis*, *S. gordonii* and biofilm formation at 12.5 μM (16). An analogue based on the DHFR inhibitor, trimetrexate (TMQ), was created that selective inhibited *S. mutans*. TMQ is currently a potent antimicrobial used in medicine as an antineoplastic and antiparasitic agent (18). TMQ has never been purposed to be used in dentistry because it can target human cells (18). Analogue #66 however, has specifically been altered to target cariogenic bacteria while keeping the key components of TMQ. In addition, Analogue #66 can be used topically as an inter-appointment medicament and not systemically to avoid any potential effects against host cells. A pilot study, compromised of MIC determinations against *S. mutants* and *S. intermedius* was carried out to determine the best concertation of analogue #66 to be used for endodontic purposes.

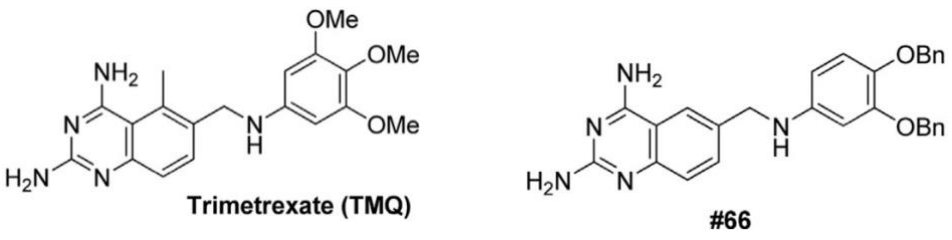


Figure 2. TMQ and Analogue #66 structure (16).

Our group has published a previous *in vitro* study that investigated the optimal concentration for the use of topical endodontic antibiotics on efficacy and their tooth discoloring effects. Antibacterial efficacy of TAP, Augmentin and tigecycline at different concentrations in a slow- release hydrogel scaffold was evaluated. In addition, discoloration potential of all 3 antibiotics (refer to figure 3) were evaluated in a hydrogel scaffold *ex-vivo* (7). TAP at 1g/mL was the most efficacious against endodontic bacterial biofilms but caused the greatest discoloration (7). Tigecycline at 1mg/mL was as efficacious as was TAP at the same concentration but caused less discoloration (7). However, this concentration had significantly less antimicrobial efficacy than TAP at 1 g/mL. Therefore, an intermediate concentration of tigecycline needs to be tested for efficacy and color stability, and for this experiment we selected 10 mg/mL to further enhance the efficacy and minimize the chances of discoloration.

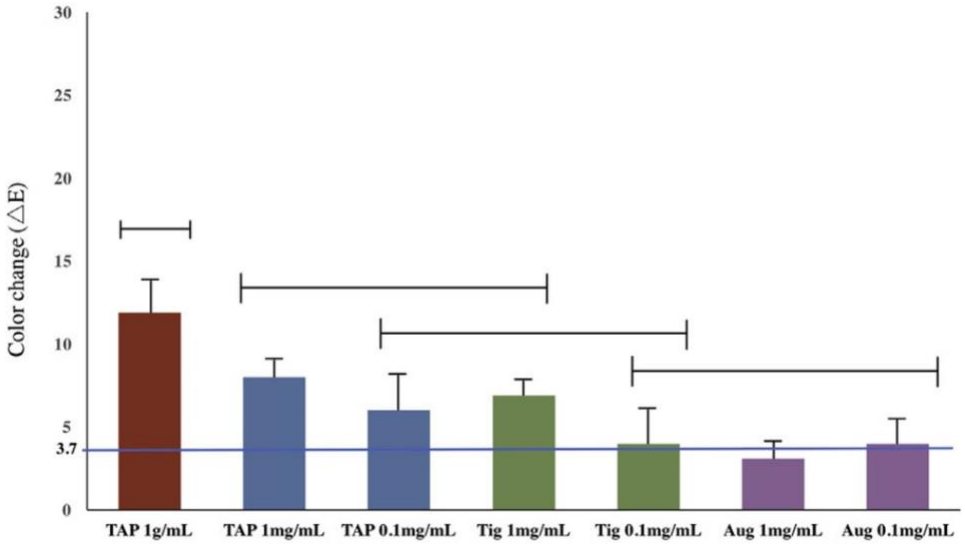


Figure 3. Comparative analysis of mean color change (ΔE), one week after treatment for TAP, tigecycline (Tig) and Augmentin (Aug). The threshold for clinical perceptibility was ΔE = 3.7 (7).

S. intermedius was chosen to be used in this experiment because it is a common virulent endodontic pathogen (19). This organism, which is considered a member of the oral microbiome, has been isolated from acute endodontic abscesses and cellulitis, (20). Furthermore, it is among the most prevalent persistent endodontic microflora following chemo-mechanical root canal preparation (21).

OBJECTIVES AND SPECIFIC AIMS

Study question: Does Analogue #66 have a more efficacious antimicrobial effect compared with calcium hydroxide and tigecycline against endodontic bacterial biofilm?

Hypothesis: Canals treated with Analogue #66 and tigecycline will have lower bacterial load compared with canals treated with, calcium hydroxide and vehicle control.

Objective 1: To determine the minimum inhibitory concentration of analogue #66 against *Streptococcus intermedius.*

Objective 2: Determine the relative efficacy of a novel antimicrobial Analogue #66 as an endodontic medicament compared with a novel antibiotic and traditional controls.

- Exposure of microbial mixture to determined concentration of #66 can be performed to test the efficacy of it as a potential anti-bacterial biofilm agent to be used as an intra-canal medicament.

MATERIALS AND METHODS

IRB approval was obtained for the use of extracted human teeth in research. A power analysis was performed, and forty single rooted teeth were collected from the periodontal and oral surgery department, University of Alabama at Birmingham (UAB) and stored in 0.5% NaOCl solution according to OSHA standards. Teeth with crown and amalgam restoration, or previously endodontically treated were not included in the study. Extracted teeth were autoclaved and placed in 5% sodium thiosulfate to inactive the effects of NaOCl.

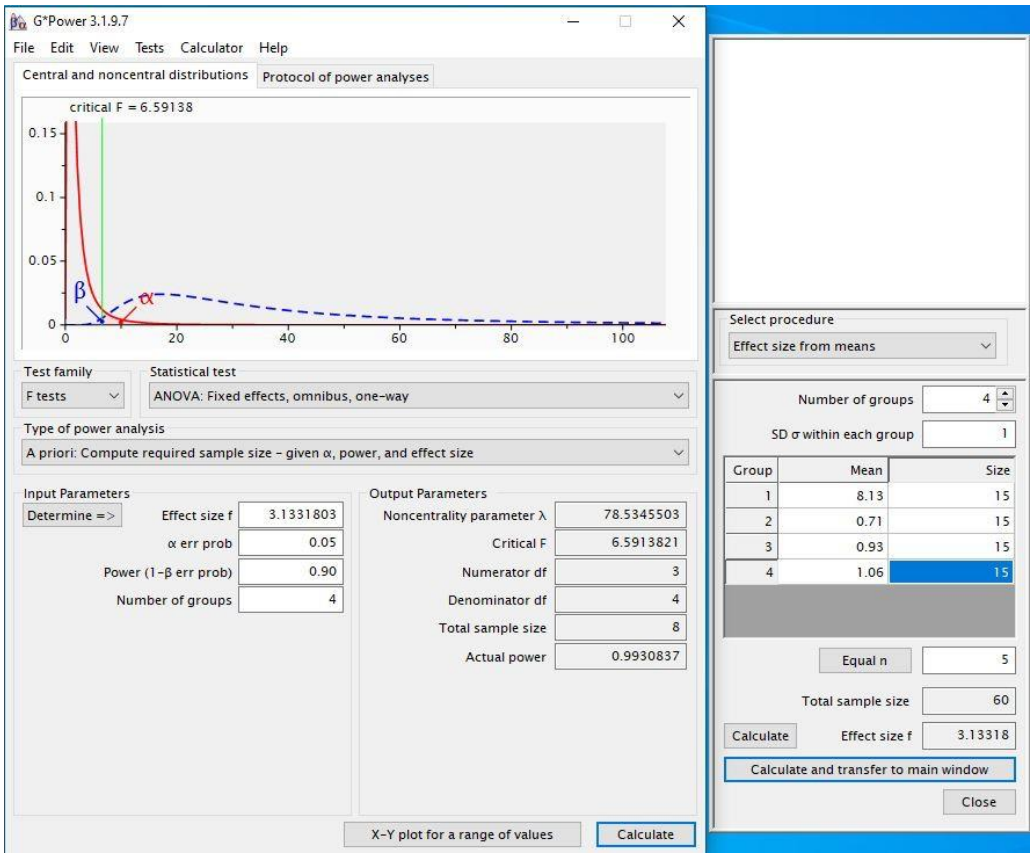


Figure 4. Power analysis of the main outcome of interest

As shown in Figure 4, obtained from the G*Power plot, which has the logarithmic means of CFUs in the control and three experimental groups, an effect size of 3.1, at an alpha error of 0.05 and Power (1-beta error) >0.9 would yield a sample of 8 per group. For consistency, we used 10 teeth per group in the experiment.

Streptococcus intermedius (ATCC 27355, USA) and *Streptococcus mutans (*ATCC 25175, USA*)* were cultured in brain heart infusion broth (Sigma-Aldrich, USA) and grown to turbidity of 1.0 McFarland density using spectrophotometer. *S. mutans* was included in the MIC experiments to act as an internal control as the MIC for this species has already been tested for MIC with analogue #66 in a previous paper (10). Brucella blood agar plates supplemented with vitamin K and iron (Thermo Scientific, USA) were used to grow *S. intermedius* and brain heart infusion agar plates (Sigma-Aldrich, USA) were used to grow *S. mutans*. Different dilutions of analogue #66 (see table 3) were incubated with either *S. mutans* or *S. intermedius* and plated with their respective agar plates and serial dilutions of 1 McFarland of the bacteria (10x, 100x, and 1000x) were performed. Plates were incubated in an anaerobic chamber with an atmosphere of 85% N2, 10% H2 and 5% CO2 at 37°C for 24 – 48 hours. CFUs were counted and recorded to determine MIC for analogue #66. A reduction in half of the CFUs of the bacteria was the threshold to determine the MIC measured in μg/mL. Three E-test strip were used according to manufacturer's instructions and the mean of the samples were taken.

Bacterial Strain	Time (in hours)
Streptococcus intermedius	24
Streptococcus mutans	24

Table 1. Incubation time for *s. intermedius* and *s. mutans*

McFarland Standard No.	0.5	1	2	4	6	8
Absorbance @600nm	0.063	0.123	0.242	0.431	0.653	0.867

Table 2. McFarland Standards and their correspondent absorbance values.

Plate number	#66	μg/mL
1	0 uM	0
2	0.5 uM	0.24
3	1 uM	0.48
4	10 uM	4.8
5	50 uM	24
6	100 uM	48
7	200 uM	95

Table 3. Experimental compound #66 values concentrations for *S. mutans* and *S. intermedius*

Forty single canal teeth were sectioned to have a standardized root length of 15mm. Canals were instrumented to the entire length, until the tip of the instrument was visible at the apical foramen without passing beyond it, using Vortex NiTi rotary instruments up to size 25/06 (Dentsply-Sirona, USA). After instrumentation, the teeth were autoclaved again to ensure complete sterilization. The root apex was sealed with sticky wax and approximately 30uL of *S. intermedius* at concentration of 3 x 10^8 colony

forming units (CFU/ml) was introduced to the canals using an irrigation syringe with Max-i-probe 30-gauge needle (Henry Schein, USA), such that the tip passively reached within 1mm of the apex. Roots were then sealed with Cavit (Henry Schein, USA). Samples were incubated with an atmosphere of 85% N2, 10% H2 and 5% CO2 at 37°C. The canals were reinoculated once a week for a total of three weeks in order for biofilm formation and maturation (22).

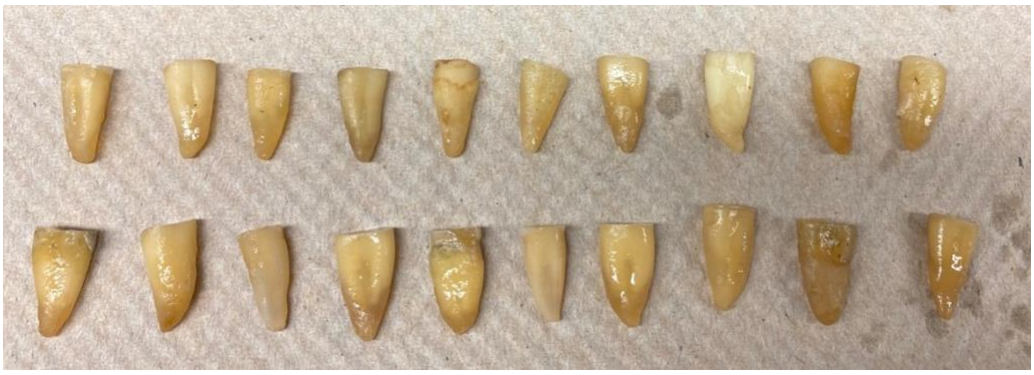


Figure 5. Image of extracted single canal teeth that were sectioned to have a standardized length of 15mm.

After biofilm formation, canals were instrumented to 40/06 to working length and irrigated with 5mL of 5% NaOCl using a Max-i-probe 30-gauge needle placed passively 1mm short of working length prior to treatment with medicaments, to simulate clinical procedures. The root canals were then irrigated with 5mL of 5% sodium thiosulfate to inactive the NaOCl then dried with paper points. The canals were filled with: (1) analogue #66 mixture (10mg/mL) in DMSO, (2) tigecycline (EMD Millipore, USA) 10 mg/mL in DMSO, (3) calcium hydroxide paste (Ultradent, USA) or (4) DMSO (Valhoma, USA) as negative control to investigate antibacterial efficacy. Compound #66 and tigecycline were dissolved in DMSO and vortexed for a minute before dispensing it. Approximately 30uL of medicament were applied to each

canal in the treatment group using a 30-gauge max-i-probe needle and BD Luer-Lock 1mL syringe (Fisher Scientific, USA). All access were sealed with Cavit and all teeth were incubated for 1 week in an anaerobic environment with an atmosphere of 85% N2, 10% H2 and 5% CO2 at 37°C.

Microbiological sampling of groups 1, 2, 3, and 4 were performed after one week. Medicament was removed via irrigating with 5mL of saline using Max-i-probe 30-gauge needle placed passively 1m short of WL. A sterile size 3 Gates Glidden bur was used throughout the canal up to the full WNL to create a slurry mixture. This mixture was collected in brain-heart fusion broth tube and vortexed for 1 minute. The resulting fluid underwent serial ten-fold dilutions (10x, 100x and 1000x dilutions), plated on enriched brucella blood agar plates, and cultured for 1 week and CFUs were counted. The materials and methodologies that were used in this experiment for microbiological sampling are based on protocols used in a previous study (5).

An exponential model was used to fit the data for *S. intermedius* and *S. mutans* to determine the concentration for 50% survival. For the extracted teeth data, one-way analysis of variance was performed, and Fisher's Least Squares Means test was used for post hoc analysis. A Chi-squared analysis was performed to compare the numbers of specimens with residual bacteria. $P\ value < 0.05$ was considered statistically significant.

RESULTS

The mean minimal inhibitory concentration (MIC) of tigecycline and analogue #66 is shown in table 4. For *S. intermedius*, the MIC against tigecycline was 0.018 μg/mL and the calculated minimal inhibitory concentration (MIC) of analogue #66 was 15.7 μg/mL. For *S. mutans*, the MIC against tigecycline was 0.25 μg/mL and the calculated minimal inhibitory concentration (MIC) of analogue #66 was 0.29 μg/mL.

Bacteria	MIC of #66 (μM)	MIC of #66 (μg/mL)	MIC of tigecycline (μg/mL)
S. mutans	0.62	0.29	0.25
S. intermedius	33	15.7	0.018

Table 4. Minimum inhibitory concentration (MIC) of analogue #66 and tigecycline for *S. mutants* and *S. intermedius*.

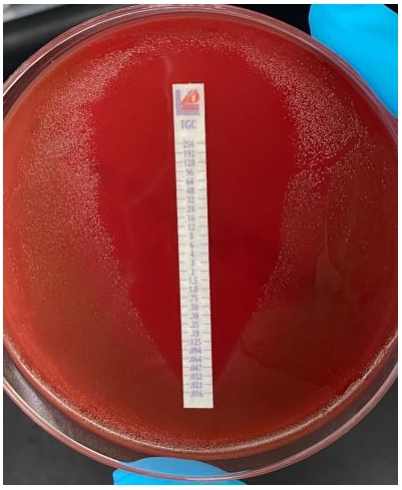
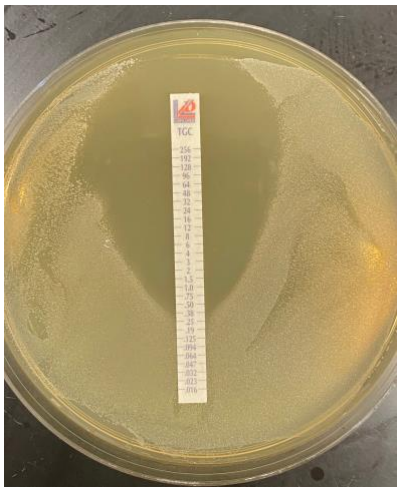

(A)

(B)

Figure 6. Example of a E-test performed on (A) *s. intermedius* and (B) *S. mutans.*

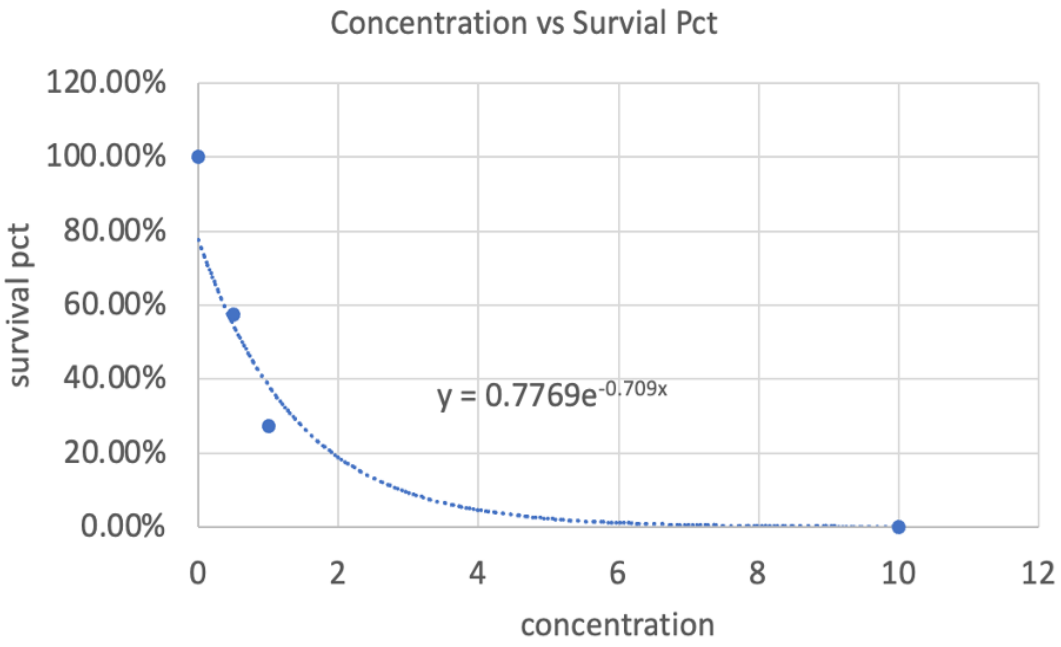


Figure 7. Exponential regression line fitted to percentage of *s. mutans* survival against varying concentration of analogue #66 showing that the concentration for 50% reduction of survival is 0.62uM.

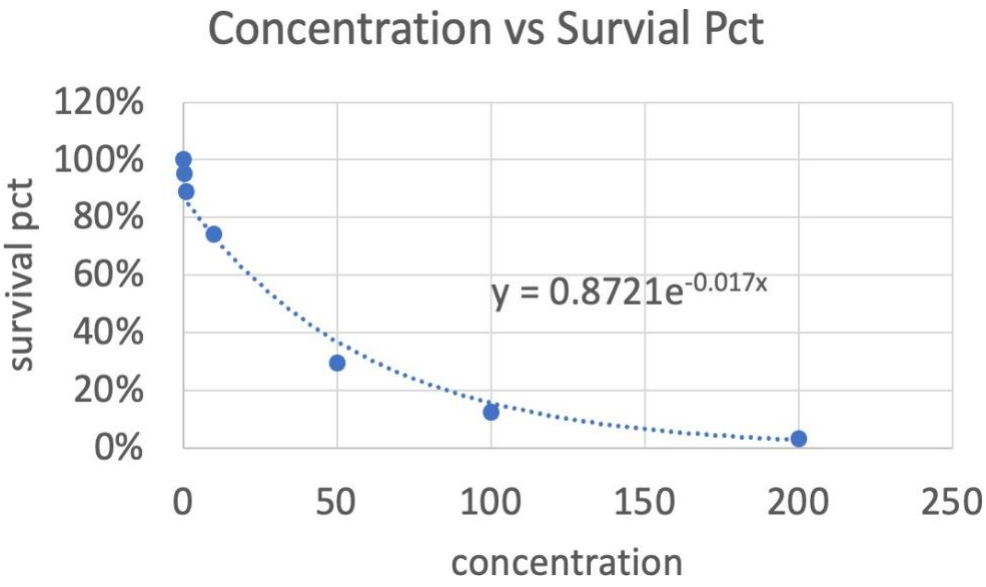


Figure 8. Exponential regression line fitted to percentage of *s. intermedius* survival against varying concentration of analogue #66 showing that the concentration for 50% reduction of survival is 33uM.

The mean bacterial survival in log CFUs/mL after each medicament is shown in Figure 9. The negative control group (DMSO) showed a mean ± standard deviation of 5.71 ± 0.17 log CFU/mL and was significantly higher than all other groups ($P < 0.05$). The log reduction for calcium hydroxide and analogue #66 was 5.07 ± 0.17 and 4.94 ± 0.15, respectively, and were not statistically significant from each other ($P > 0.05$). The tigecycline group (0.58 ± 1.22) had significantly less residual CFUs compared with all other groups ($P < 0.05$). In addition, the Chi- squared analyses comparing the number of specimens with residual bacteria (figure 5) showed a statistical significance ($P < 0.05$).

	positive	negative
DMSO	10	0
CH	10	0
#66	10	0
Tigecycline	2	8

Table 5. Raw data of the 4 groups showing either the presence (positive) or absence (negative) of residual bacteria on plates.

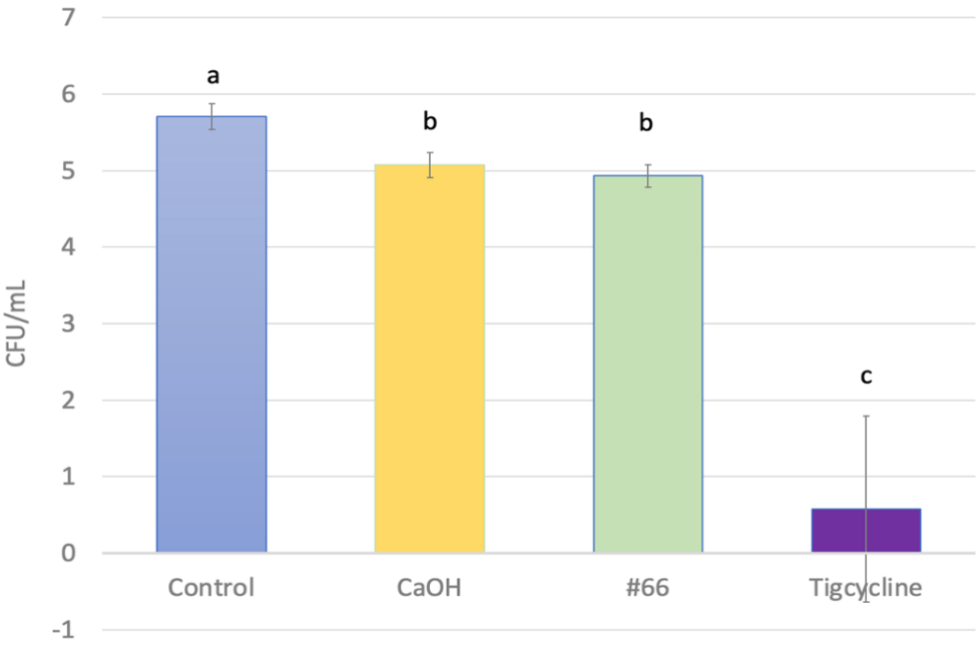


Figure 9. The log concentration of CFUs (log CFU/mL) following different medicament placement on 3-week-old *S. intermedius* biofilm. Different letters above the bars indicate a statistically significant differences between the groups ($P < 0.05$).

DISCUSSION:

Trimetrexate is used as an antimicrobial medication for *Pneumocystis carinii*, a rare fungal infection that affected AIDS patients before an effective treatment for HIV was identified. The dose that is used in medicine is currently 50 mg/mL (23). Tigecycline is commonly delivered IV, at a dose of 50mg/mL, for serious abdominal or skin infection (24). While the MIC is useful for treating bacteria in their planktonic form, in our experiment, we are dealing with a biofilm that would be resistant to the MIC concentration hence having the need to increase it.

Results from the MIC portion of this experiment is comparable with previous findings from Dr. Velu's lab regarding analogue #66 efficacy against *S. mutans*. Dr. Velu's lab showed that the MIC for *S. mutans* was 0.78 μM and the MIC from our experiment showed a value of 0.62 μM (16). The similarity between the two values acts as a quality control to check and confirm efficacy of agent #66 on *S. mutans* in this model system.

After mechanical instrumentation with NaOCl, the negative control group in our study still resulted in a high number of residual bacteria. Many studies have shown that after cleaning and shaping with NaOCl or other irrigants, it is common to still culture high amounts of residual bacteria (25). In addition, studies have reported streptococci as one of the most cultured species post-instrumentations (26). This reinforces the fact that intracanal medicaments are useful to improve disinfection.

The ability of calcium hydroxide to eradicate bacterial species from the root

canal is questionable and has raised a lot of debates throughout literature. For example, McGurkin-Smith et al. showed that after intra-appointment application of calcium hydroxide, there was a statistically significant reduction in mean bacterial count, with only 14% of the teeth detected bacteria (27). However, an *ex vivo* study by Haapasalo et al. showed that dentine can inactivate the antibacterial activity of calcium hydroxide (28). In addition, a clinical study by Peters et al. also showed that the number of canals positive for bacteria increased after placing calcium hydroxide medicament (29). The result from this study is consistent with the argument that calcium hydroxide is not effective enough as an intra-appointment medicament, indicating the need to develop a more effective medicament. Analogue #66 showed promising results from the MIC experiment, however in the extracted teeth, analogue #66 showed no difference from calcium hydroxide. This could be because in the MIC experiment, planktonic bacteria were plated whereas in the extracted teeth, a biofilm was formed for three weeks prior to the canal preparation and medication. While analogue #66 was useful in treating planktonic bacteria, the *S. intermedius* biofilm formed in the extracted teeth became more resistant to it.

Tigecycline showed the highest bacterial reduction in all 4 samples. The mean bacterial survival in log CFUs/mL for Tigecycline at 10mg/mL was 0.58 according to our findings, compared with the mean log CFUs/mL for Tigecycline at 1mg/mL which was 0.93 according to AlSaeed's paper (7). As expected, increasing the concentration of Tigecycline resulted in a reduction in mean bacterial survival, however, this increase is only warranted if this does not result in significant discoloration to the crown. Future studies are needed to investigate esthetics concerns with Tigecycline at 10mg/mL. Tigecycline is a member of tetracyclines and has been

shown to be highly effective against bacteria from necrotic root canals (30). The advantage of using Tigecycline over Tetracycline and Minocycline is that it overcomes the two major mechanisms of Tetracycline resistance, making it an ideal medicament for resistant infections (31). In addition, Tigecycline is a better choice than TAP as an intracanal medicament because at the same concentration, Tigecycline showed the same efficacy against common endodontic microbes as TAP but with less discoloration (7).

CONCLUSION:

Within the limitations of this study, all three of the tested chemicals, calcium hydroxide, analogue #66 and tigecycline showed significant antimicrobial efficacy against *S. intermedius* biofilm. Tigecycline was shown to be the most effective against *S. intermedius* biofilm while analogue #66 was shown to be the same effectiveness as calcium hydroxide.

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APPENDIX

IRB APPROVAL LETTER

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Office of the Institutional Review Board for Human Use

470 Administration Building
701 20th Street South
Birmingham, AL 35294-0104
205.934.3789 | Fax 205.934.1301 |
irb@uab.edu

NHSR DETERMINATION

TO: Fouad, Ashraf F

FROM: University of Alabama at Birmingham Institutional Review Board
Federalwide Assurance # FWA00005960
IORG Registration # IRB00000196 (IRB 01)
IORG Registration # IRB00000726 (IRB 02)
IORG Registration # IRB00012550 (IRB 03)

DATE: 22-Jan-2021

RE: IRB-300006559
Use of extracted anonymous human teeth in in vitro research

The Office of the IRB has reviewed your Application for Not Human Subjects Research Designation for the above referenced project.

The reviewer has determined this project is not subject to FDA regulations and is not Human Subjects Research. Note that any changes to the project should be resubmitted to the Office of the IRB for determination.

if you have questions or concerns, please contact the Office of the IRB at 205-934-3789.

Additional Comments:

~ 150 extracted, de-identified teeth from SOD clinics for Endodontic research