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作品名稱　Eradicating Cystic Fibrosis Biofilms by a Novel Non-Toxic, Multi-Pathway Salicylate Therapy

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Eradicating Cystic Fibrosis Biofilms by a Novel Non-Toxic, Multi-Pathway Salicylate Therapy

An (Ann) Wang

1. Introduction

1.1. Cystic Fibrosis Biofilms

Biofilms are bacterial aggregates in a matrix of polysaccharides, proteins and nucleic acids (Donlan, 2002). They account for 80% of all chronic infections and cause over 500,000 deaths annually. Cystic fibrosis (CF) is a genetic disorder characterized by mucus accumulation in the respiratory tracts (Morrison et al., 2020). This impairs mucociliary clearance, allowing chronic colonization by bacterial biofilms, leading to fatal respiratory failure, lung scarring, and necrosis of pulmonary epithelial tissues (Martin et al., 2021).

1.2. Obstacles in Current Treatments

Three major therapies are used against CF biofilms: (1) aminoglycoside antibiotics like tobramycin, (2) non-aminoglycoside antibiotics such as ciprofloxacin and vancomycin, and (3) non-antibiotic therapies including flushing, chlorination, and ultraviolet disinfection. These have two major flaws. First, they are cytotoxic; 30% of patients experience acute kidney injury after three days of intravenous aminoglycoside therapy (Joyce et al., 2017). Furthermore, non-aminoglycoside therapies can cause phospholipid buildup in lysosomes of proximal tubule epithelial cells, accounting for 10-20% of acute renal failure cases. Second, antibiotic resistance due to horizontal gene transfer and mutations has significantly reduced treatment effectiveness. Therefore, cystic fibrosis biofilms remain a critical threat with few effective treatments.

1.3. Salicylate Derivatives

This project tackled this issue using an innovative non-antibiotic approach with salicylate derivatives. Salicylates, a class of benzoic acids—benzene-based carboxylic acids (Figure 1)—used in painkillers and blood thinners, were investigated for their antibiofilm potential through a 3-step process:

1. **Literature review:** Identified three key biofilm therapeutic targets: quorum sensing, bacterial adhesion, and cell motility. Disrupting these pathways would result in biofilm eradication.
2. **Molecule Identification:** Recognized key molecules in each pathway: LasR, adhesins, and flagellin. Inhibiting these molecules would disrupt the pathways.
3. **Screening:** Found that salicylates could inhibit the identified molecules, though they had never been tested against cystic fibrosis biofilms.

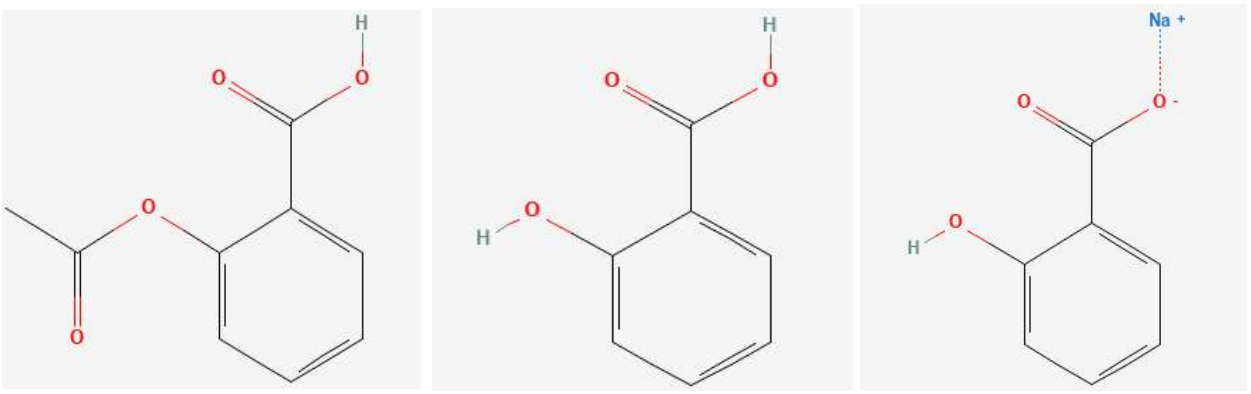

Figure 1: Structural formulas of (left) acetylsalicylic acid, (centre) salicylic acid, and (right) sodium salicylate

2. Research Purpose

Considering the antibiofilm potential of salicylates, I posed the question: "How can salicylates, both alone and in combination with antibiotics, form the first effective treatment for cystic fibrosis biofilms?" I hypothesized the following:

1. Salicylates will reduce CF biofilms by inhibiting bacterial adhesion, quorum sensing, and cell motility.
2. Salicylates will outperform clinically relevant antibiotics due to the prevalence of antibiotic resistance.
3. Salicylates will act synergistically with clinically relevant antibiotics to enhance treatment efficacy.

3. Procedure

3.1. Constants and Variables

3.1.1. Bacterial Strains and Culture Conditions

The most common CF bacterial strains, *Pseudomonas aeruginosa* (PA14) (ATCC 15442) and methicillin-sensitive *Staphylococcus aureus* (MSSA, Rosenbach) (ATCC 25904) were used. Overnight cultures were incubated at 37˚C with gentle shaking in 5 mL of 50% lysogeny broth (LB) for PA14 and in 5 mL of 100% tryptic soy broth (TSB) and 1% D-glucose for MSSA.

3.1.2. Antimicrobial Treatments

Three types of salicylates were compared against three relevant antibiotics. Stock solutions of sodium salicylate (NaSA), vancomycin (VAN), ciprofloxacin (CIP) and tobramycin (TOB) were prepared by dissolving in sterile water (320 mM, 12 µg/mL, 8 µg/mL, and 20 µg/mL respectively). Salicylic acid (SAL) and acetylsalicylic acid (ASA) were dissolved in 100% ethanol (320 mM) for higher solubility. Solvent toxicity tests established that ethanol has no statistically significant effect on biofilm disruption ($p < 0.01$).

3.2. Phase I: Single Drug (Individual) Testing

3.2.1. Planktonic and Biofilm Individual Treatment Assays

Overnight cultures were diluted to an optical density (OD) of 0.05 at 600 nm. Then, 200 µl of bacterial suspension was inoculated in triplicate in a 96-well polystyrene microtitre plate (Figure 2). Stock antimicrobial solutions were diluted 2:1 and applied after 0 hours in inhibition assays and after 48 hours in eradication assays. The plate was incubated at 37˚C for planktonic growth and biofilm maturation. After 48 hours of treatment, planktonic cells and nutritionally depleted medium were aspirated, and the wells were rinsed three times with distilled water. Results are averaged from three replicates of three trials.

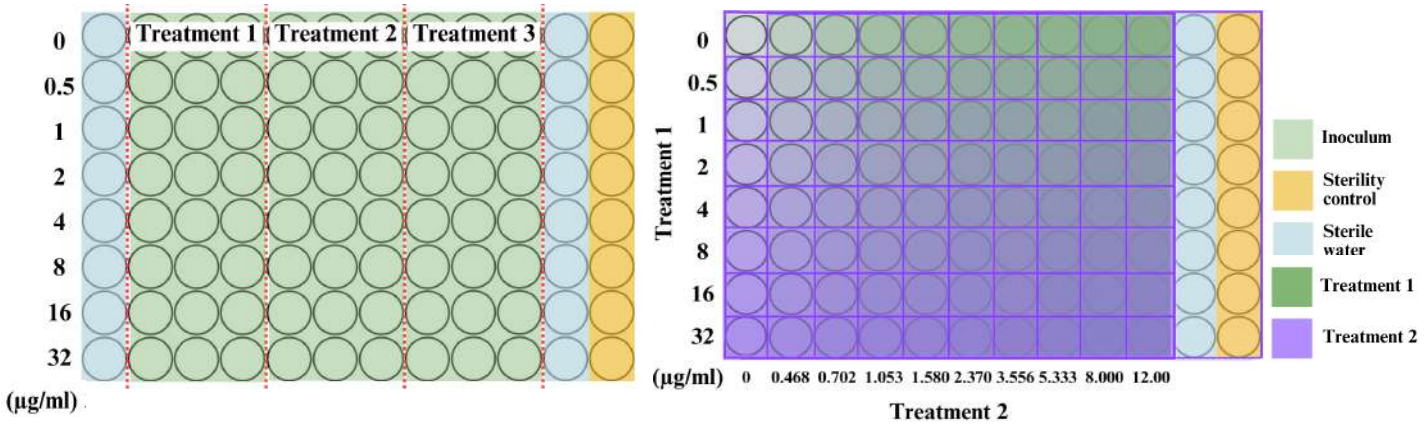

Figure 2: (Left) Sample 96-well microplate layout for biofilm inhibition and eradication assays. Only the interior wells of the plate are used to mitigate the possibility of evaporation from wells. The exterior wells are filled with sterile water or serve as a sterility control for the media of interest. (Right) Layout of checkerboard plate; gradients of drugs along X & Y axes

3.2.2. Data Collection Method 1: Staining

Two types of stains were used: crystal violet (CV) to measure biomass reduction and tetrazolium chloride (TTC) to measure cell death. Following staining, OD_{600} and OD_{595} measurements were taken with an Epoch Microplate Spectrophotometer. Biomass was calculated relative to untreated wells (100% biomass) and the sterility control (0% biomass), yielding MIC_{50} and MIC_{90} (minimum inhibitory concentration) values, the lowest antimicrobial concentrations that reduce biomass by more than 50% and 90% (R.E.W. Hancock Lab, 2023).

3.2.2. Data Collection Method 2: Scanning Confocal Laser Microscopy

Scanning confocal laser microscopy (SCLM) is an optical imaging technique that captures 2D slices of a sample to create a 3D model (Figure 3). Samples were stained with the light-sensitive nucleic acid stain SYTO-9 and propidium iodide to indicate the live-dead status of bacterial cells. At 25x magnification (numerical aperture = 0.075), lasers pass through a 35 µm pinhole, blocking out-of-focus light and increasing resolution. Laser power was set at 2.0%, with detector voltages at 780 V (green), and 800 V (red); this balances signal strength, blocking

of background signals, and preventing laser burns on the sample (Le et al., 2018). Close-up views at 200x magnification (numerical aperture = 0.8 with apochromatic objective to correct for chromatic operation) used 1.8% laser power and detector voltages of 650 V (green) and 700 V (red). In 3D (Z-stack) mode, slices were 2 µm apart vertically, with eight times pixel averaging.

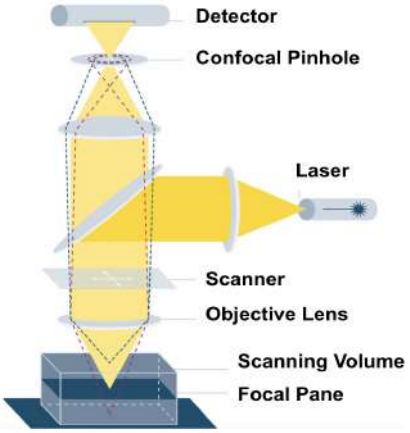

Figure 3: Principle of scanning confocal laser microscopy; increased focus relative to wide-field fluorescence microscopy.

3.3. Phase II: Multiple Drug (Synergy) Testing

Synergy is when the interaction of multiple drugs leads to greater eradication than the sum of their individual effects.

3.3.1. Checkerboard Biofilm Assays

Salicylic acid (SAL) was paired with TOB and CIP for PA14, and with VAN and CIP for MSSA. Serial dilutions created two concentration gradients per plate: one for the antibiotic and one for the salicylate along the X and Y axes (Figure 1**)**. These gradients were immediately applied to the inoculum in inhibition assays and after 48h of static growth in eradication assays. TTC and CV staining were used for eradication, while only CV staining was used for inhibition, as described in Section 3.2.2.

3.3.2. Combination Therapy Index (CTI)

Synergy is evaluated using the Combination Therapy Index (CTI), a bespoke statistic developed for this experiment. The CTI compares the combined effects of drugs to the best individual drug effects at the same concentration, relative to the cumulative effects of both drugs **(**Figure 4**).** Unlike previous methods like fractional inhibitory concentrations (FICs), which rely on threshold killing measurements (e.g., MIC50), the CTI accurately represents the complex dose-response curves of biofilms, including irregular subinhibitory biofilm simulation peaks and incomplete eradication. The CTI uses the percentage of biomass disrupted (K_i), defined as the percentage of growth under treatment subtracted from the average of untreated samples. It is compatible

with the commonly used checkerboard assay layout, and can evaluate multiple antimicrobials, making it an accurate, novel metric for assessing biofilm multidrug interactions.

$$CTI = 2\frac{\kappa_{combi} - \max\{\kappa_a, \kappa_b\}}{\kappa_a + \kappa_b} \qquad CTI = n\frac{\kappa_{combi} - \max\{\kappa_a, \kappa_b \ldots \kappa_n\}}{\sum_{i=1}^{n} \kappa_i}$$

Figure 4. CTI formula for 2 antimicrobials (left), CTI formula for *n* antimicrobials (right).

4. Results

4.1. Salicylates Independently Reduced PA14 Biofilms

All three salicylates outperformed the untreated control (Figures 5 & 6), confirming that salicylates can effectively reduce PA14 biofilms. They achieved 40-50% non-toxic eradication, indicating the potential for synergy, as antibiotics often struggle to eliminate the last 20-30% of a biofilm at their highest non-toxic concentrations (Sharma et al., 2019). Without complete eradication, biofilm remnants can regenerate within 48 hours. The addition of salicylates to antibiotic treatments could prevent this. The efficacy of salicylates was biphasic (circled in red), likely due to both cell killing and inhibition of the biofilm matrix. This observation is crucial for understanding their mechanism of action.

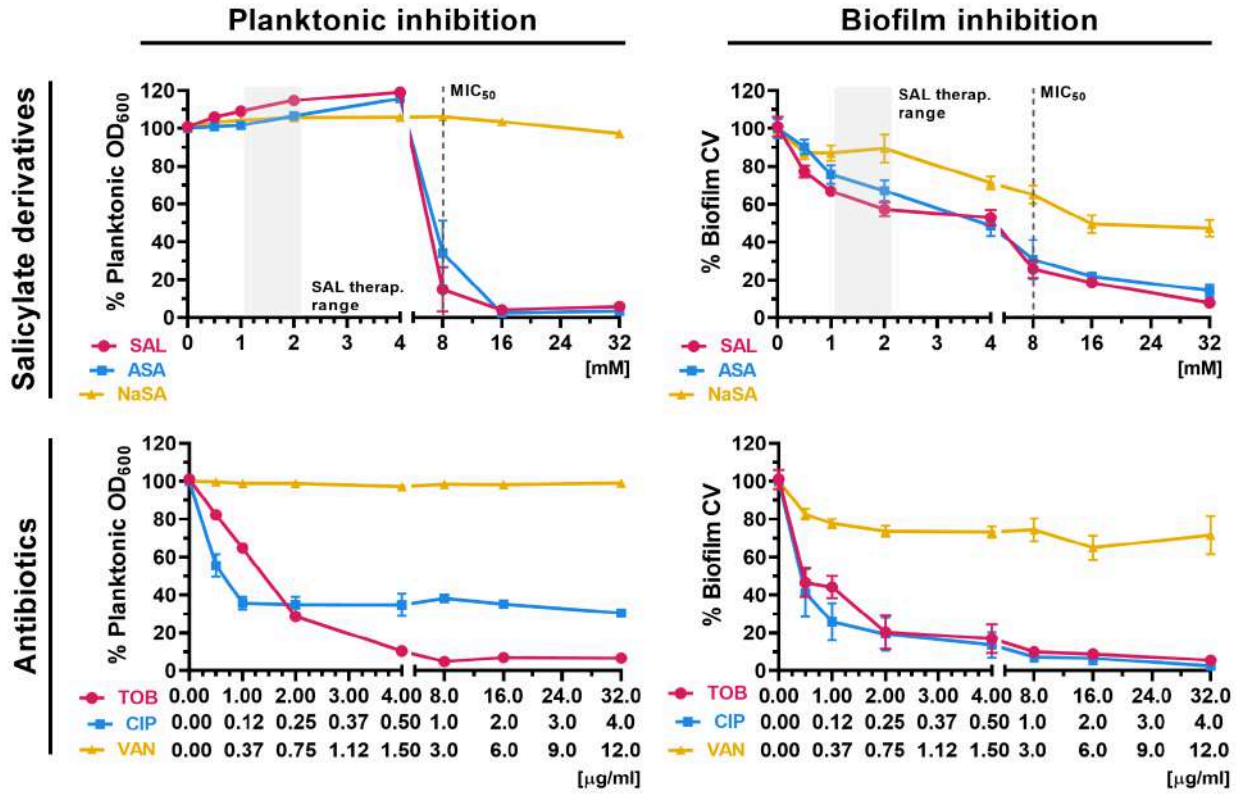

Figure 5. Individual PA14 inhibition efficacy graphs.

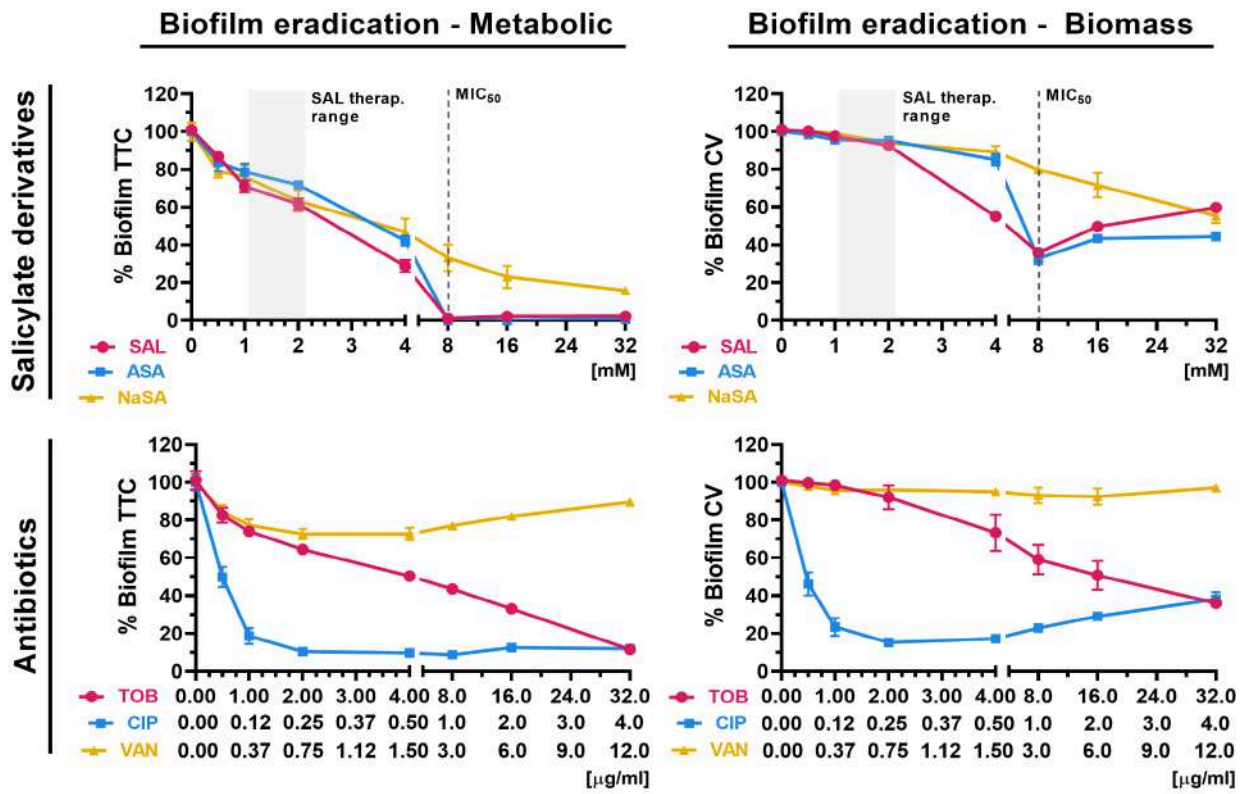

Figure 6. Individual PA14 eradication efficacy graphs.

4.2. Salicylates Independently Outperformed Antibiotics in MSSA

Salicylates outperformed the untreated control and achieved nearly 100% non-toxic eradication (see Figures 7 & 8). Salicylic acid (SAL) was the most effective salicylate, eradicating an average of 98.7% in biomass at only 1 µg/mL. This was verified by an extra sum of squares F-test (Tables 1, 2, 3), where p-values lower than 0.05 suggested that one treatment had outperformed another. In contrast, antibiotics increased biofilm growth, likely due to subinhibitory biofilm stimulation, where antibiotic-induced stress exacerbates chronic infections. Thus, salicylates were independently effective and outperformed clinically relevant antibiotics.

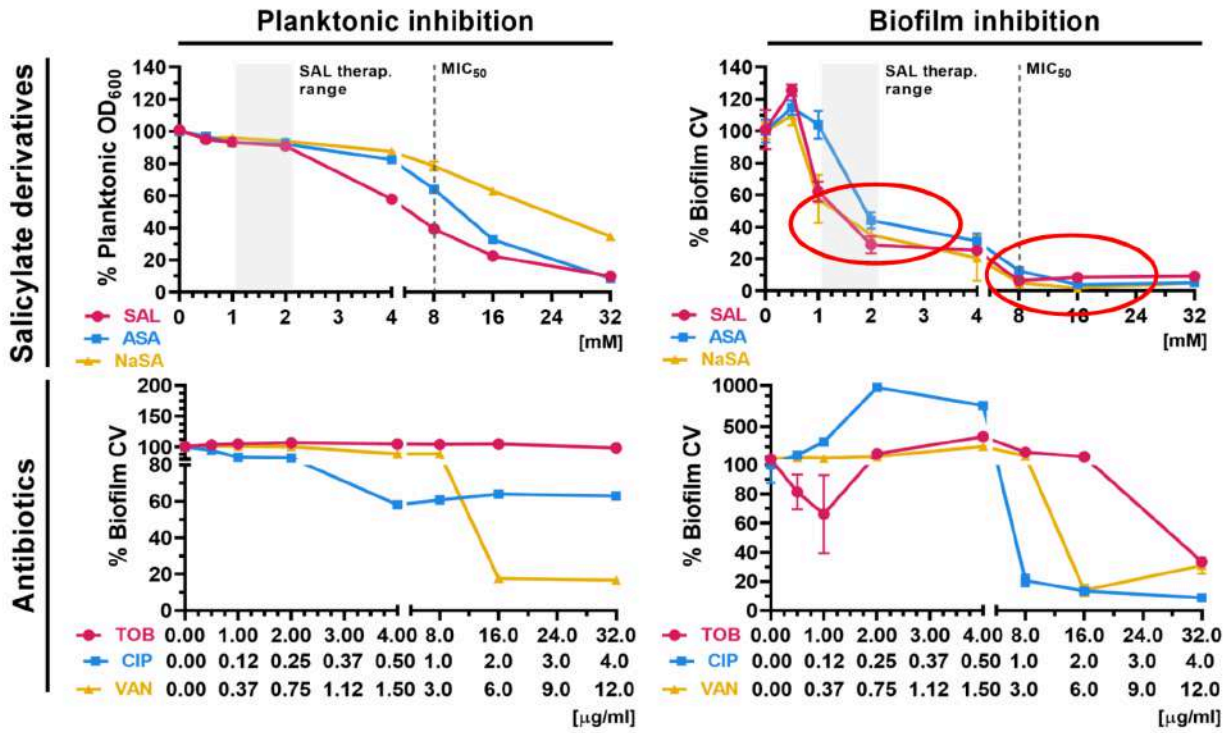

Figure 7. Individual MSSA inhibition efficacy graphs.

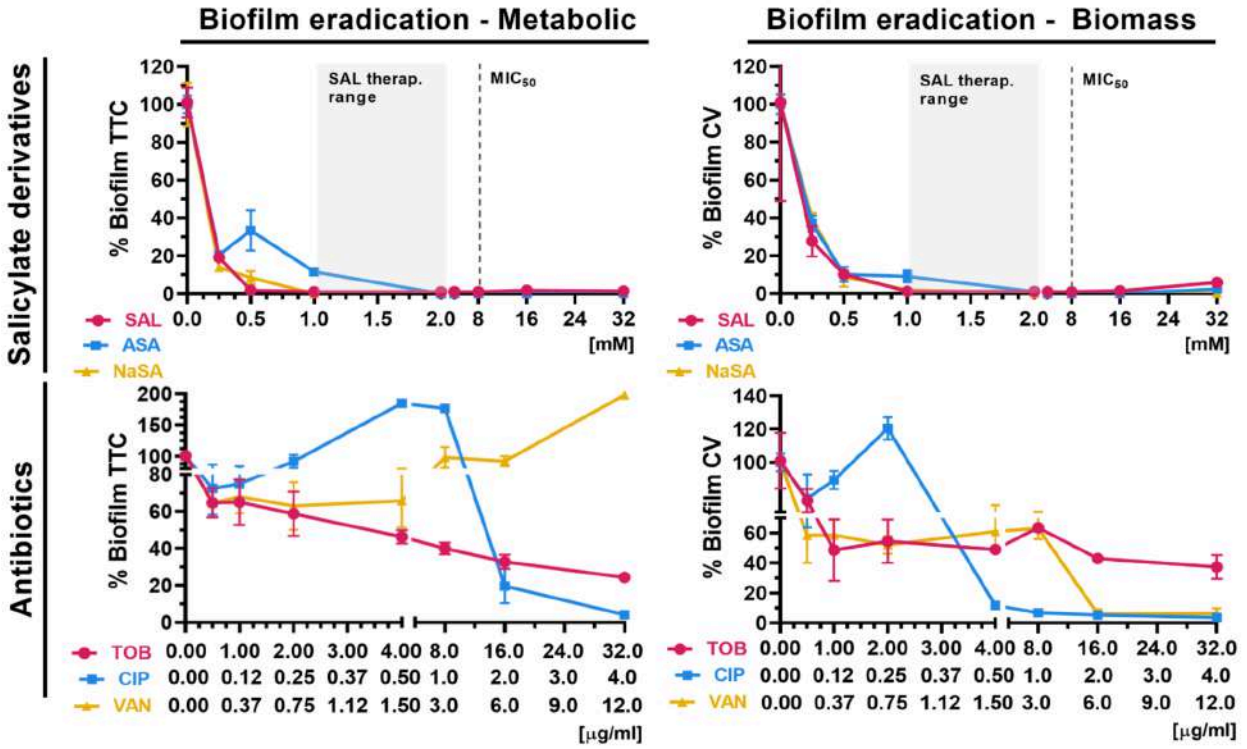

Figure 8. Individual MSSA eradication efficacy graphs.

Comparison	F-Statistic	p-value
Salicylic vs. Acetylsalicylic acid	9.877	1.180×10^{-5}
Salicylic vs. Sodium salicylate	1.782	0.1515
Acetylsalicylic acid vs. Sodium salicylate	3.072	0.0268

Table 1: Results of Extra-Sum of Squares F-Test (MSSA Inhibition)

Comparison	F-Statistic	p-value
Salicylic vs. Acetylsalicylic acid	91.2565	2.8039×10^{-7}
Salicylic vs. Sodium salicylate	20.3991	0.0003
Acetylsalicylic acid vs. Sodium salicylate	117.6824	9.1887×10^{-8}

Table 2: Results of Extra-Sum of Squares F-Test (MSSA Eradication, CV)

Comparison	F-Statistic	p-value
Salicylic vs. Acetylsalicylic acid	37.51	5.42^{-6}
Salicylic vs. Sodium salicylate	17.56	0.000162
Acetylsalicylic acid vs. Sodium salicylate	24.59	3.71^{-5}

Table 3: Results of Extra-Sum of Squares F-Test (MSSA Eradication, TTC)

4.3. Salicylates are Anti-Synergistic in PA14 Biofilms

The CTI colour scale (see Figures 9-A & 10-A) indicates anti-synergy, as blue areas suggest that adding salicylic acid decreased the performance of TOB and CIP. This anti-synergy may result from interference between the mechanisms of salicylates and antibiotics. For instance, ciprofloxacin's inhibition of DNA gyrase may affect the ability of salicylates to inhibit quorum-sensing molecules. However, since the salicylic acid-CIP combination is effective in MSSA, this explanation only holds for salicylic acid-TOB. A more universal explanation is that PA14 is less responsive to combination treatments, which accounts for the reduced performance.

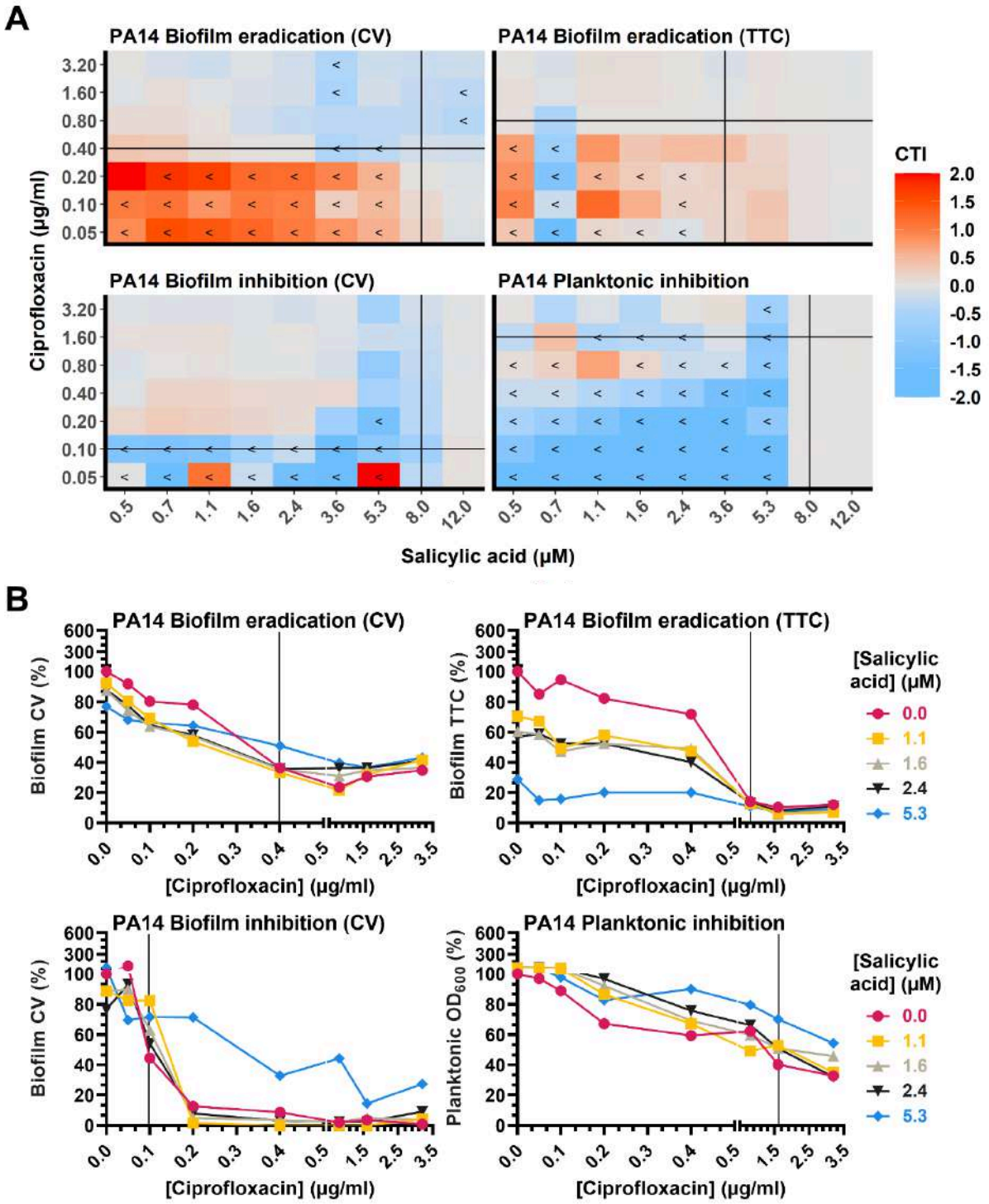

Figure 9. **A**) CTI values for salicylic acid-ciprofloxacin in PA14, black lines indicate 50% inhibition/eradication for the individual treatments. (**B**) inhibition/eradication treatments at select salicylic acid concentrations. Fractional inhibition/eradication concentrations are provided when both drugs are present and only when the concentration of salicylic acid is ≤ MIC_{50}/$MBIC_{50}$/$MBEC_{50}$.

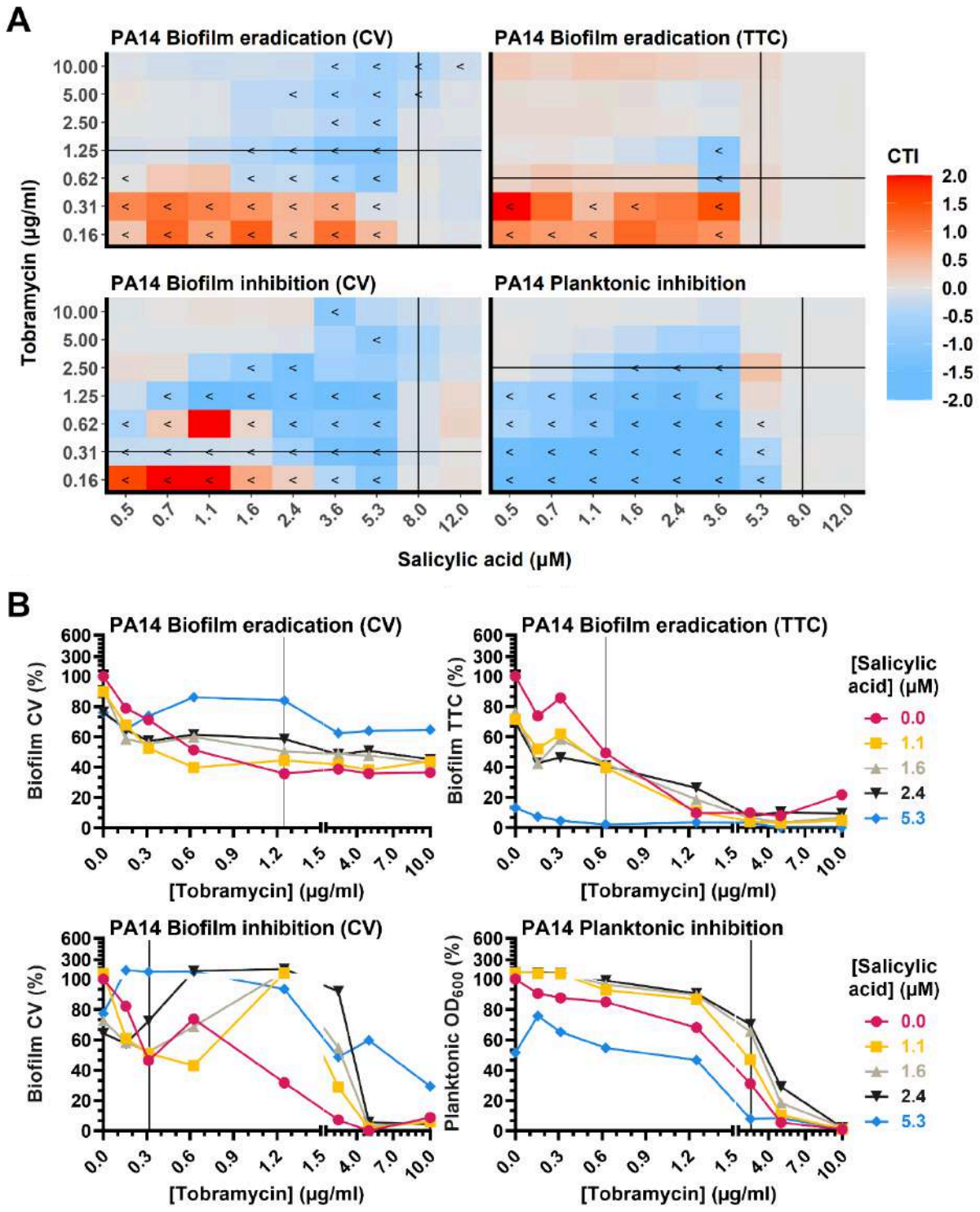

Figure 10. Synergy PA14 salicylic acid-tobramycin graphs (See Fig. 7 for full explanation).

4.4. Salicylates are Synergistic in MSSA Biofilms

The CTI colour scale (see Figures 11-A & 12-A) indicates synergy, as red areas suggest that adding salicylic acid increased the performance of VAN and CIP. For example, adding 2.37 mM of salicylic acid to VAN increased eradication from 77% to 98%. This synergy may result from the alignment of mechanisms between

VAN and salicylic acid. For instance, VAN's inhibition of bacterial cell wall synthesis might expedite the inhibition of bacterial adhesion proteins by salicylic acid. Additionally, the resources spent fighting one drug could make MSSA more vulnerable to another. The efficacy of SAL-antibiotic combinations indicates that a multidrug approach is a promising direction for future therapies.

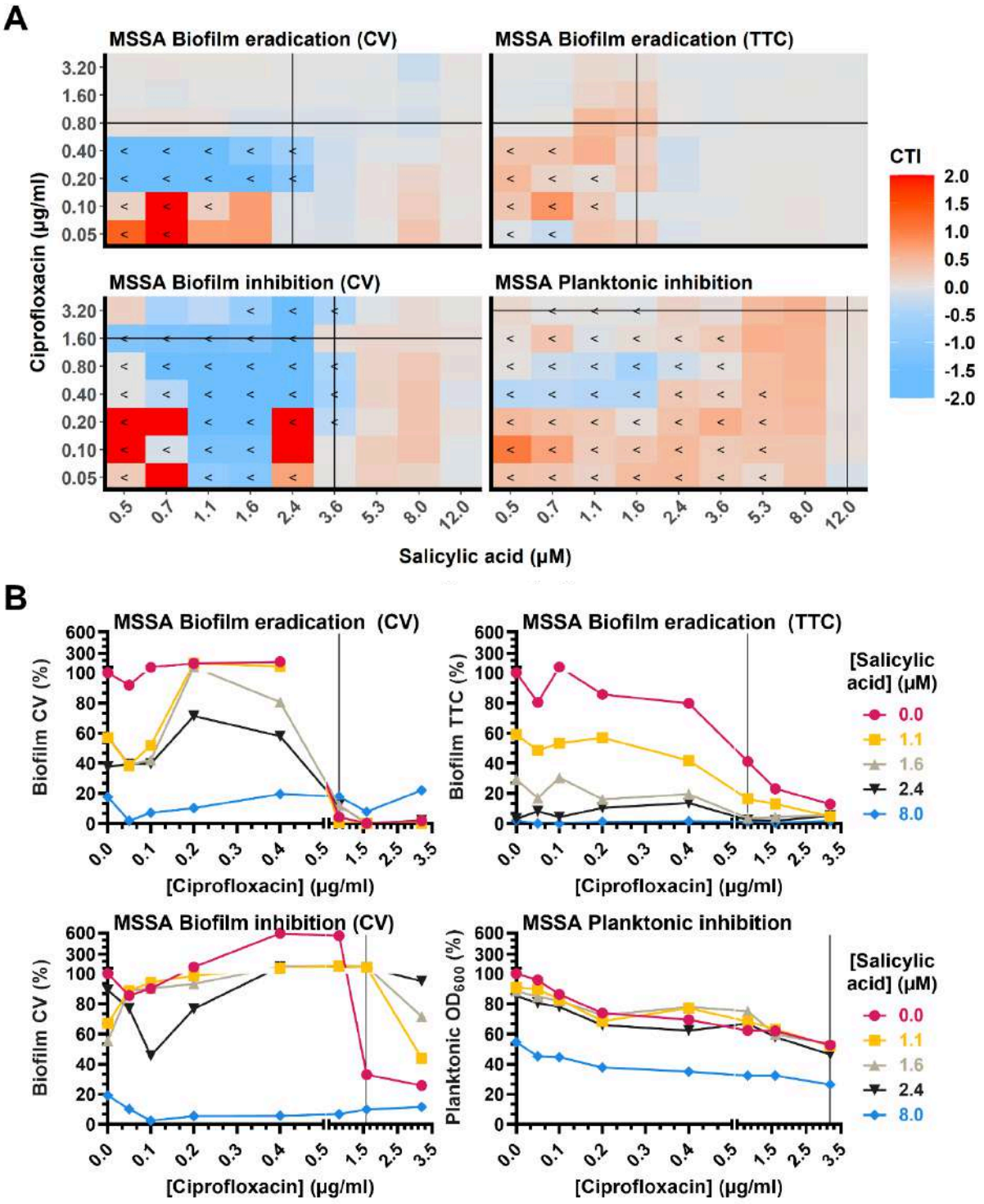

Figure 11. Synergy MSSA salicylic acid-ciprofloxacin graphs (See Fig. 7 for full explanation).

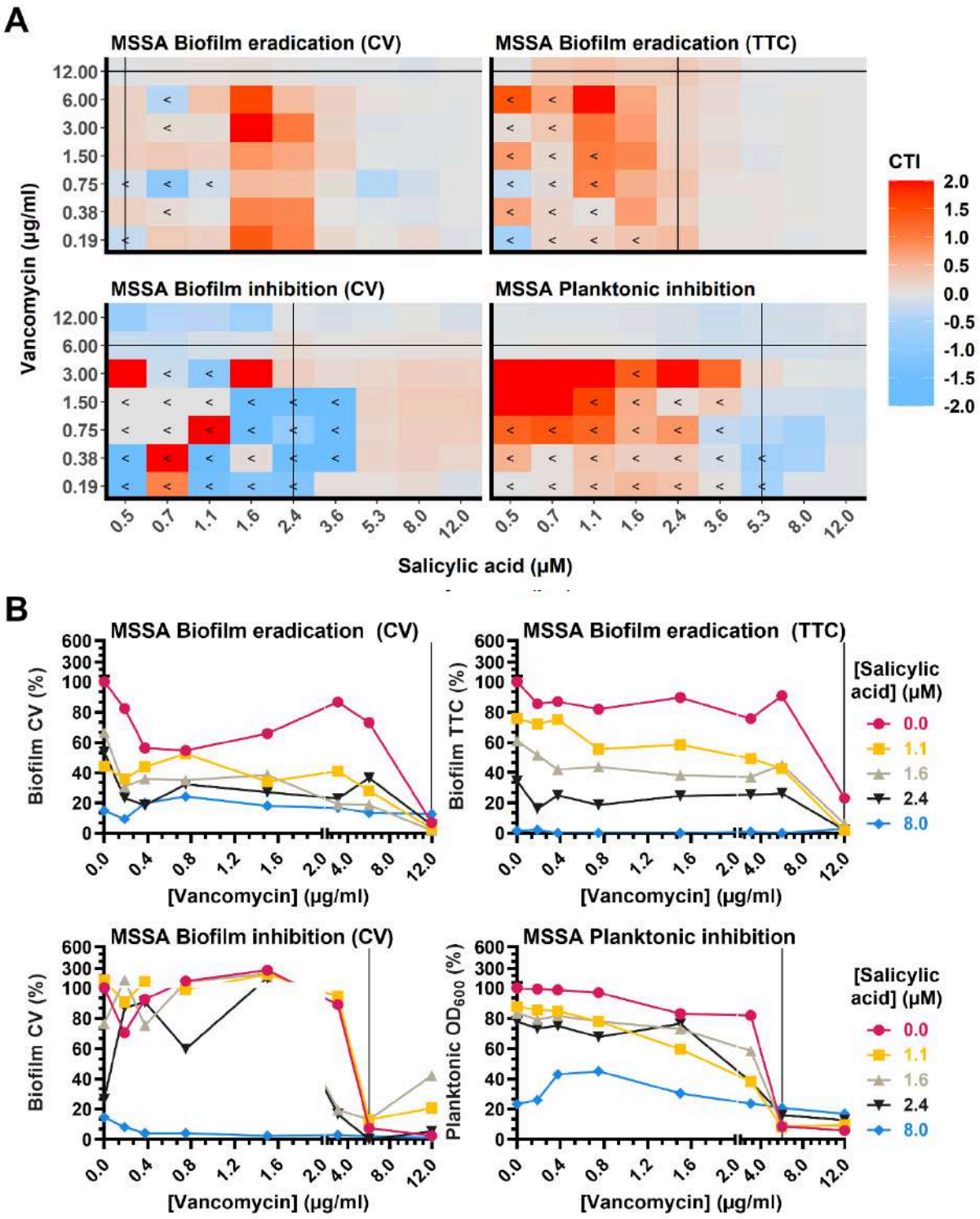

Figure 12. Synergy MSSA salicylic acid-vancomycin graphs (See Fig. 7 for full explanation).

4.5. Scanning Confocal Laser Microscopy Analysis

SCLM images were evaluated by probe colour, bacterial sparseness, and biofilm thickness. The prevalence of red cells in salicylate-treated biofilms (see Figure 13) suggests that SAL effectively kills cells. In comparison, tobramycin-treated biofilms were predominantly green, indicating TOB's ineffectiveness in killing cells. Empty

spaces in the salicylate-treated biofilm suggest that SAL also effectively reduces the biofilm matrix. Additionally, orthogonal views showed that salicylates reduced biofilm thickness. Thus, SCLM imaging demonstrates that salicylates are effective in biofilm eradication.

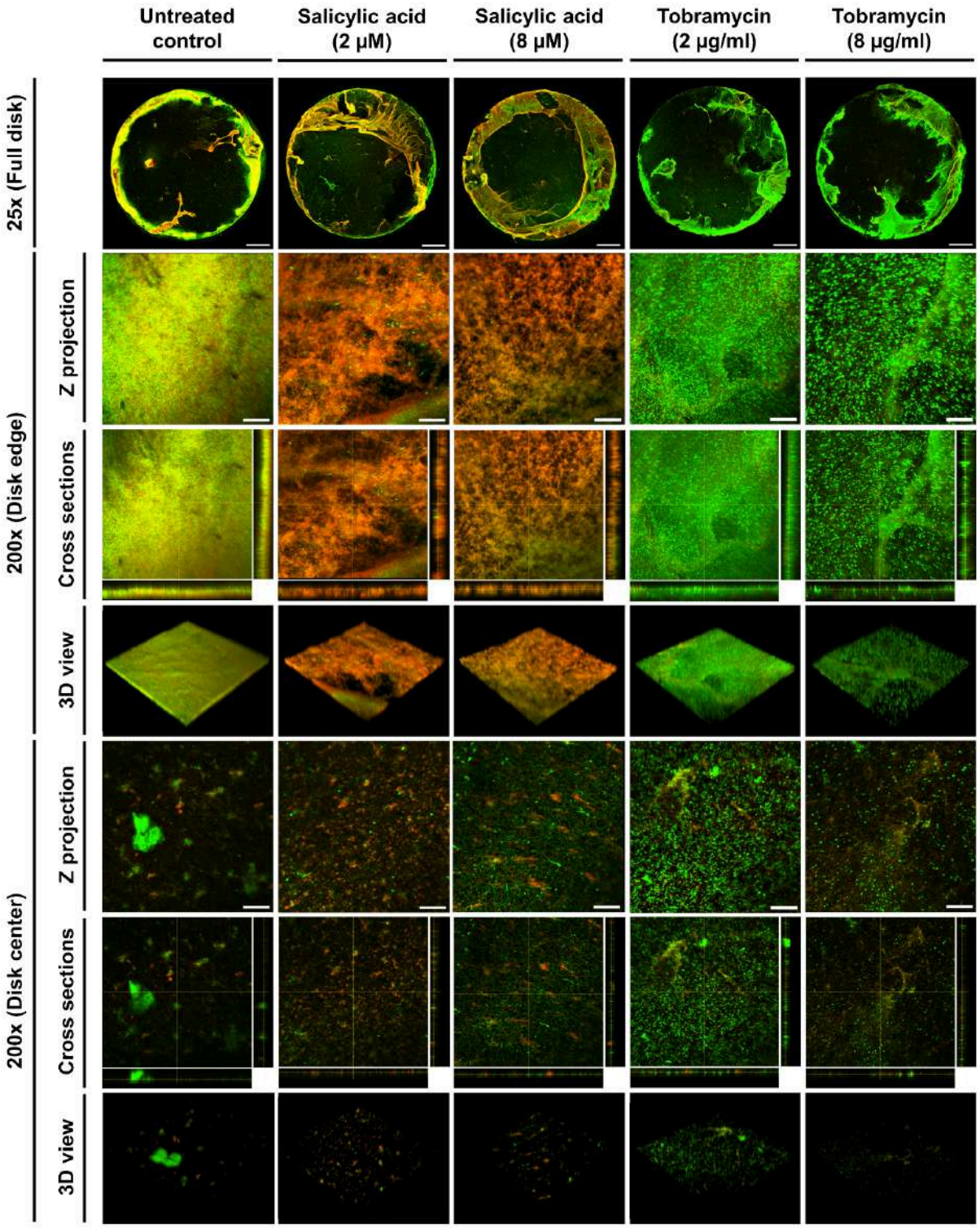

Figure 13. PA14 SCL microscopy under 25x and 200x conditions; 2D, 3D, and orthogonal views.

5. Statistical Analysis

5.1. False Discovery Rate (FDR) Correction

To mitigate the increased likelihood of false positives due to multiple comparisons, a Bonferroni correction was applied, generating a stricter threshold of significance. The Bonferroni test sets k as the number of comparisons ($k = 45$ for single drug testing, $k = 720$ for multidrug testing), and a as the desired p value ($a = 0.01$). The adjusted significance thresholds are $a_{adjusted} = 0.0002$ (single drug testing) and $a_{adjusted} = 1.3 \times 10^{-5}$ (multidrug testing).

5.2. Tests of Normality

To apply parametric tests like a t-test or ANOVA, the dataset must follow a normal distribution. This was assessed using a Q-Q plot and the Shapiro-Wilk test. For a portion of the data, the Q-Q plot showed deviations from the identity line (y = x), indicating that the data were not normally distributed. The Shapiro-Wilk test confirmed this, as these samples had p-values below the alpha level ($a = 0.01$) (Table 4), leading to rejection of the null hypothesis ($H_0 = X \sim N(\mu, \sigma^2)$). For those samples, (Table 4), a t-test or ANOVA was not suitable. For another portion of the data, the Shapiro-Wilk test yielded p-values greater than 0.01. Here, a t-test was suitable.

Drug	Era/Inh	Stain	Test Statistic	p-value
Tobramycin	Eradication	TTC	0.7649	3.57×10^{-5}
		CV	0.8157	0.0003
	Inhibition	CV	0.8510	0.0023
Ciprofloxacin	Eradication	TTC	0.8639	0.0022
		CV	0.8840	0.0059
	Inhibition	CV	0.7611	0.0073
Vancomycin	Eradication	TTC	0.8613	0.0019
		CV	0.8805	0.0049
	Inhibition	CV	0.8040	0.0003
Salicylic Acid	Eradication	TTC	0.5864	4.38×10^{-7}
		CV	0.5184	2.60×10^{-8}
	Inhibition	CV	0.8167	0.0006
Acetylsalicylic Acid	Eradication	TTC	0.7242	2.14×10^{-5}
		CV	0.6231	3.89×10^{-7}
	Inhibition	CV	0.8427	0.0016
Sodium Salicylate	Eradication	TTC	0.7651	0.0081
		CV	0.5989	2.01×10^{-7}
	Inhibition	CV	0.8239	0.0007

Table 4: Shapiro-Wilk Dataset Test Results (MSSA), $p < 0.01$ (red)

5.3. Unpaired 2-Tailed T-Tests

Therefore, for the normally distributed samples, two-tailed unpaired t-tests were conducted. The effects of salicylates were statistically significant, with p-values less than 0.0002 for individual samples and p-values below 1.3×10^{-5} for synergy testing.

5.4. Extra Sum of Squares F-Test

For non-normally distributed samples, a nonlinear curve regression fit (dose-response or biphasic dose response) followed by an extra sum of squares F-test was used. However, this method requires normally distributed residuals ($r_i = y_i - \hat{y}_i$). A Q-Q plot and the Shapiro-Wilk test verified residual normality. The Q-Q plot showed minimal deviations from the identity line, visually confirming normal distribution. Additionally, the Shapiro-Wilk test produced *p*-values exceeding the alpha level of 0.01, meaning the null hypothesis of normal distribution could not be rejected (Table 5)**.** Thus, the residuals were normally distributed, validating the use of a nonlinear curve regression fit. This approach reduces the number of multiple comparisons needed.

Drug	Era/Inh	Stain	Test Statistic	p-value
Tobramycin	Eradication	TTC	0.8897	0.0131
		CV	0.94	0.62
	Inhibition	CV	0.9856	0.9853
Ciprofloxacin	Eradication	TTC	0.9556	0.2916
		CV	0.8014	0.0212
	Inhibition	CV	0.8671	0.1411
Vancomycin	Eradication	TTC	0.9391	0.1160
		CV	0.9814	0.8919
	Inhibition	CV	0.8121	0.0385
Salicylic Acid	Eradication	TTC	0.9146	0.0293
		CV	0.9310	0.0731
	Inhibition	CV	0.9097	0.0347
Acetylsalicylic Acid	Eradication	TTC	0.9610	0.4597
		CV	0.9438	0.1514
	Inhibition	CV	0.9554	0.3533
Sodium Salicylate	Eradication	TTC	0.96	0.77
		CV	0.8910	0.2041
	Inhibition	CV	0.9381	0.1481

Table 5: Shapiro-Wilk Post-Regression Residual Test Results (MSSA), $p > 0.01$

Following a nonlinear curve regression fit (Table 6), an extra sum of squares F-test was used to compare treatments. Initially, all treatments were compared against the untreated control. Subsequently, comparisons were made among the salicylates, and between individual and combination treatments to determine if adding a second drug enhanced the first's performance. In MSSA, salicylic acid was the most effective, while SAL and

acetylsalicylic acid were not statistically different in PA14. All salicylates outperformed the untreated control, and in MSSA, all salicylates significantly outperformed all antibiotics.

Drug	Era/Inh	Stain	Bottom	Top	IC50	Hill Slope
Tobramycin	Eradication	TTC	0.0483	0.9564	1.9726	2.4134
		CV	0.11	1.00	0.24	4.17
	Inhibition	CV	−1.30	1.552	7.680	0.365
Ciprofloxacin	Eradication	TTC	0.3001	1.0272	0.3641	7.3732
		CV	-0.6330	0.9910	1.9010	0.4367
	Inhibition	CV	1.998	0.072	1.898	-35.207
Vancomycin	Eradication	TTC	0.3077	1.1105	3.5992	7.7059
		CV	-471.9442	0.9941	4.8418×10^{18}	0.1507
	Inhibition	CV	19080.84	1.184	20.57	4.778
Salicylic Acid	Eradication	TTC	0.1706	1.1407	0.1066	4.6267
		CV	0.1987	2.7373	0.1579	3.2240
	Inhibition	CV	0.3421	1.8794	0.2587	4.4510
Acetylsalicylic Acid	Eradication	TTC	0.1571	1.1424	0.1604	2.4272
		CV	0.2087	2.9183	0.1936	3.3026
	Inhibition	CV	0.3533	2.0961	0.3511	5.6570
Sodium Salicylate	Eradication	TTC	0.10	1.22	0.24	1.12
		CV	0.1829	2.2758	0.2373	7.3463
	Inhibition	CV	0.2812	2.1848	0.2743	3.2450

Table 6: Non-Linear Curve Regression Fit Results (MSSA)

6. Conclusions and Project Impact

6.1. All Hypotheses Supported

This project developed an effective, non-toxic, and economical therapy for cystic fibrosis biofilms. The efficacy is likely due to the inhibition of bacterial adhesion, quorum sensing, and cell motility, as described in Section 1.3. The treatment is non-toxic within the human therapeutic range, and economical, with salicylates being on average 3.4 times cheaper than aminoglycoside antibiotics.

6.2. Clinical Applications

The use of this therapy is feasible as inhalant and intravenous applications of salicylates are already in clinical use. Future applications could include nebulizing salicylates via pressurized metered-dose inhalers, dry powder inhalers, or medical nebulizers for aerosol-based delivery. Such delivery systems are already used for TOB, including the TOBI® Podhaler®. Aerosolization of salicylates can target endobronchial infection sites, minimizing systemic toxicity.

6.3. Multidrug Resistance

The combination of salicylates with antibiotics can combat multidrug-resistant (MDR) strains for two reasons. First, bacteria using resources to fight one drug become more susceptible to the second treatment. Second,

resistance often depends on random mutation, and it is statistically less for bacteria to develop simultaneous resistance to two treatments. Thus, salicylate-antibiotic therapies can help reduce the spread of antibiotic resistance.

6.4. Challenges and Limitations

There were two major challenges in experimentation. First, biofilm thickness was insufficient, initially measuring around 200 μm instead of the average 500 μm found in cystic fibrosis airways. This was addressed by adjusting the media to 100% TSB and adding 1% glucose to enhance biofilm growth. The second challenge was the toxicity of salicylates, particularly to the stomach lining and the tympanic membrane. This issue can be mitigated by controlled administration in clinical settings and targeted drug delivery systems.

6.5. First Synergy Formula to Evaluate Biofilm Growth

The Combination Therapy Index (CTI) developed in this project is applicable to all biofilm infections beyond cystic fibrosis, including those in the bloodstream, urinary tract, and bones, many of which use combination therapies. The CTI simplifies evaluation by eliminating the need for 3D graphs and is applicable beyond biofilms, including in cancer, metabolic, and inflammatory disorders.

6.6. Comparisons to Existing Research

This project aligns with existing research in several ways. Similar to Kunin et al. (1995), sodium salicylate decreased PA14 biofilms, likely by inhibiting flagellin production. Additionally, as noted by Wu et al. (2000), salicylates have broad therapeutic effects, from Kawasaki disease to cystic fibrosis. However, these results differ from Lagadinou et al. (2020), who suggested salicylates were only effective in MSSA at concentrations above the human therapeutic range. This project demonstrated non-toxic efficacy against MSSA biofilms.

7. FUTURE DIRECTIONS

7.1. Cytotoxicity Assays

To establish the therapeutic range for combination treatments and confirm the non-toxicity of salicylates, cytotoxicity assays on A549 human alveolar epithelial cells are necessary. Two staining methods can be used: trypan blue, which stains compromised cell membranes and allows for cell counting by hemocytometer, and CellTox™ Green Dye, which binds to the DNA of cells with compromised membranes, enabling real-time measurements via fluorometer.

7.2. Cell Motility Tests

Plate-based assays on liquid or low-viscosity agar can verify the impact of salicylates on cell motility. Observing the swarming and swimming patterns of PA14 can provide insights. Cell motility testing is crucial because the ability of biofilms to initiate permanent host attachment requires regulation of flagellar rotation.

7.3. Multispecies Biofilms

Cystic fibrosis biofilms often consist of multiple species, with MSSA microcolonies growing embedded in a PA14 biofilm. Optimizing environmental conditions for the coexistence of PA14 and MSSA, achievable with the use of Dulbecco's Modified Eagle Medium and bovine serum albumin (Cendra et al., 2019), is essential. Testing salicylates on multispecies samples may enhance the applicability of this therapy for CF patients.

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1. Novelty and Significance:

   This project addresses a critical medical issue - the treatment of cystic fibrosis (CF) biofilms. The approach of using salicylates, both alone and in combination with antibiotics, represents a novel strategy for combating these persistent infections. The significance of this research is underscored by the high mortality rate associated with biofilm infections and the limitations of current treatments.

2. Strengths:

   The study exhibits creativity in proposing salicylates as an alternative to traditional antibiotics, addressing the issues of cytotoxicity and antibiotic resistance.The project has potential real-world applications, aiming to develop an effective, non-toxic, and economical therapy for CF biofilms.

3. Weaknesses:

   The study lacks in vivo testing on animal models or human subjects, which limits the assessment of the treatment's efficacy and safety in real-world conditions. There is no comparative analysis with existing treatments, making it difficult to evaluate the relative benefits of the proposed

therapy. The project could benefit from clearer figure legends and more detailed explanations of experimental procedures, such as the time required for biofilm formation and the process of biofilm degradation during drug treatment.The economic analysis comparing salicylates to aminoglycoside antibiotics could be more comprehensive, including factors such as production costs and potential side effects.