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Enhancing Accumulation and Penetration Efficiency of Next-Generation Antibiotics to Mitigate Antibiotic Resistance in *Pseudomonas aeruginosa* PAO1

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ABSTRACT

This study explores the efficacy of advanced antibiotic compounds against *P. aeruginosa*, focusing on Antibiotic B, an enhanced derivative of Ceftriaxone. The study measured the intracellular uptake of Antibiotic B and introduced a novel adjuvant, Influximax, which augmented its antibacterial activity. Results showed a diminished potential for resistance emergence with Antibiotic B, particularly when used in combination with Influximax. The study suggests that optimizing antibiotic delivery into bacterial cells and leveraging synergistic adjuvant combinations can enhance drug resistance combat.

1. INTRODUCTION

Antibiotic Resistance: A Global Health Imperative

The escalating issue of antibiotic resistance constitutes a formidable threat to global public health, demanding a concerted effort from the scientific community, healthcare providers, policymakers, and the public at large. The Centers for Disease Control and Prevention (CDC) estimates that in the United States alone, antibiotic-resistant pathogens are responsible for 2.8 million infections annually, leading to over 35,000 deaths [1]. The World Health Organization (WHO) has also declared antibiotic resistance as one of the top 10 global public health threats facing humanity [2]. Such resistance compromises our ability to

combat infectious diseases and undermines many advances in health and medicine. These sobering statistics are a clarion call for immediate action to combat this crisis.

The Mechanisms of Resistance

Antibiotic resistance occurs through various mechanisms, including genetic mutations and the acquisition of resistance genes via horizontal gene transfer. The misuse and over-prescription of antibiotics, coupled with inadequate diagnostics, have accelerated this natural process, allowing resistant strains to thrive, and spread. The emergence of multi-drug resistant (MDR), extensively drug-resistant (XDR), and even pan-drug-resistant (PDR) pathogens has introduced new challenges within clinical settings. Infections caused by these resistant strains are associated with higher morbidity, mortality, and healthcare costs due to the limited efficacy of existing antibiotics [3].

Tackling Antibiotic Resistance through Multifaceted Strategies

Innovations in Antibiotic Development

The relentless nature of bacterial evolution calls for an equally dynamic approach to antibiotic development. New antibiotics that can circumnavigate the resistance mechanisms of formidable pathogens like *P. aeruginosa* PAO1 are under intensive research. However, the discovery of new drugs is not sufficient. Adjuvants that inhibit the resistance pathways of bacteria are also being investigated to enhance the potency of existing antibiotics [4].

Innovations in Drug Delivery

The future of antibiotic development may lie in the advancement of drug delivery mechanisms. Nanotechnology promises to revolutionize the field by offering vehicles for antibiotics that can improve their stability, penetration, and targeted delivery [5]. Such nanocarriers have the potential to protect the antibiotic agents from premature degradation, enhance their solubility, and provide sustained release, which could prove pivotal in overcoming resistance.

Biofilm Disruption

P. aeruginosa PAO1's propensity to form biofilms represents a considerable challenge in treatment, as these biofilms shield the bacterial colonies from both antibiotics and the host immune system. Compounds that can disrupt biofilm integrity and enhance antibiotic penetration are therefore a significant area of research [6]. Biofilm disruptors could expose bacterial cells to immune defenses and antimicrobial agents, rendering them more susceptible to treatment.

An Integrated Approach to Antibiotic Resistance on *Pseudomonas aeruginosa* PAO1

The intricate and multifarious nature of antibiotic resistance necessitates an integrated approach that combines novel drug development with enhancements to existing treatments and the prudent application of antimicrobial agents. This strategy must be global, involving international cooperation and the sharing of knowledge and resources.

Antibiotic resistance has become one of the paramount challenges in modern medicine, demanding a reinvigorated approach to antibiotic development and deployment. Among the pantheon of resistant organisms, *Pseudomonas aeruginosa* PAO1 exemplifies a formidable adversary, epitomizing the intrinsic and acquired resistance mechanisms that define the resilient nature of pathogenic bacteria [7]. This Gram-negative pathogen's ability to thrive in diverse and hostile environments is indicative of its versatile metabolic capabilities and its complex armory of resistance determinants, including efflux pumps, enzyme production, and biofilm formation [8].

Recent trends in antibiotic discovery have shifted towards the refinement of existing antibiotics and the enhancement of their effectiveness through improving drug accumulation and penetration [9]. The rationale behind this strategy stems from the recognition that higher intracellular concentrations of antibiotics are critical in overcoming the constitutive defenses of *P. aeruginosa* PAO1. These defenses often manifest as reduced permeability of the bacterial outer membrane and active expulsion of antimicrobial agents, rendering many conventional antibiotics ineffectual [10].

Research has consistently shown that the accumulation of antibiotics within bacterial cells is a pivotal determinant of their bactericidal efficacy. This is particularly relevant in the context of *P. aeruginosa* PAO1, where the dense and impermeable outer membrane constitutes a significant barrier to antibiotic entry [11]. Concurrently, the penetration rate of antibiotics is equally critical. The ability of an antibiotic

to rapidly traverse the cell envelope and reach its target is a key factor in circumventing bacterial defense mechanisms that are time-dependent, such as the induction of efflux pumps and the expression of antibiotic-degrading enzymes [12].

Considering this, novel strategies that can enhance the accumulation and penetration of antibiotics hold promise for surmounting the formidable obstacle of drug resistance in *P. aeruginosa* PAO1. Next-generation antibiotics and adjuvants are being explored to this end, with the latter specifically aimed at impeding bacterial resistance mechanisms, thereby facilitating greater antibiotic uptake and retention [13]. For example, inhibitors of efflux pumps have been shown to significantly increase the intracellular concentration of various antibiotics, suggesting a synergistic approach to antibiotic therapy [14].

Furthermore, advancements in nanotechnology offer innovative methods to improve drug delivery. Nanocarriers can protect antibiotics from degradation, enhance their diffusion through biological barriers, and target them more precisely to the site of infection [15]. This targeted approach not only maximizes the therapeutic effects of antibiotics but also minimizes the exposure of non-target bacteria, potentially reducing the selection pressure for resistance [16].

Another promising direction is the exploration of the role of biofilm disruptors. Since *P. aeruginosa* PAO1 is known to form biofilms, which confer an additional layer of protection against antibiotics, agents that can destabilize biofilms may enhance antibiotic penetration and effectiveness [17]. The disruption of biofilm architecture exposes bacteria to immune clearance and facilitates the deeper penetration of antimicrobial agents, addressing one of the critical challenges in treating *P. aeruginosa* infections [18].

The severity of the antibiotic resistance crisis underscores the need for an integrated approach that combines new drug discovery with the optimization of existing therapeutic agents. This strategy should be comprehensive, involving not only chemotherapeutic innovation but also stewardship programs to prolong the effectiveness of newly optimized antibiotics [19]. As research progresses, the goal remains clear: to refine the tools in our armamentarium to ensure they remain sharp against the evolving threat of bacterial pathogens like *P. aeruginosa* PAO1.

2. MATERIALS AND METHODS

2.1. Bacterial Strains and Culture Conditions

The study utilized the *Pseudomonas aeruginosa* PAO1 strain obtained from the American Type Culture Collection (ATCC 15692). Bacterial cultures were grown in Lysogeny Broth (LB) at 37˚C and agitated at 200 rpm until reaching the mid-logarithmic phase. For antibiotic exposure, cultures were diluted to an optical density at 600 nm (OD600) of 0.1, which correlates to approximately 1 × 10^8 colony-forming units (CFU)/mL. This standardization ensured a consistent starting point for all antibiotic treatment assays [20]. To optimize *P. aeruginosa* PAO1 growth, several modifications were introduced:

1) Adjusted Ion Concentration: The sodium chloride content in LB medium was tailored to meet *P. aeruginosa* PAO1's unique ion requirements, ensuring an osmotic balance conducive to its optimal growth.

2) pH Optimization: The LB medium's pH was finely tuned to a slightly alkaline range (between 6.5 and 7.5), aligning with *P. aeruginosa* PAO1's preferred pH range and closely simulating physiological conditions.

3) Supplements: Additional supplements, including trace elements and, when necessary, extra carbon or nitrogen sources, were added to LB medium to mimic host organism conditions, promoting the growth and expression of virulence factors in *P. aeruginosa* PAO1.

4) Temperature and Agitation: Maintaining an incubation temperature of 37˚C, akin to human body temperature, and agitating at 200 rpm ensured optimal growth conditions for the aerobic *P. aeruginosa*, crucial for its pathogenic nature.

The significant of the modifications was to mimic *P. aeruginosa* physiological conditions in human hosts to promote robust bacterial growth and preserve infection characteristics, enhancing the study's relevance in real-world therapeutic challenges.

2.2. Next-Generation Antibiotic Compounds

Next-generation antibiotics were sourced from a collaboration with a leading pharmaceutical company specializing in antibiotic development. These antibiotics were chosen based on their novel mechanisms of action, which include disruption of bacterial cell wall synthesis, protein synthesis inhibitors, and nucleic acid synthesis inhibitors. The compounds were solubilized according to the manufacturer's specifications to ensure optimal activity [21]. In this research, Antibiotic A was solubilized in a slightly acidic solution and stored at 4˚C to prevent degradation, while Antibiotic B was dissolved in sterile water or physiological saline, used fresh due to its limited stability, with both antibiotics prepared according to manufacturer's specifications, including adjustments for concentration, pH balance, and storage conditions, ensuring their efficacy in experimental assays against *Pseudomonas aeruginosa* PAO1.

2.3. Assay for Antibiotic Accumulation

For the quantification of antibiotic accumulation in our study, a fluorescence-based assay was utilized, wherein each antibiotic was tagged with specific fluorescent markers to enable their detection and quantification upon uptake by bacterial cells.

1) Antibiotic A was conjugated with a fluorescein isothiocyanate (FITC) derivative, a widely used fluorescent tag that binds covalently to the antibiotic molecule without significantly altering its antibacterial properties. FITC was chosen for its high fluorescence efficiency and stability under biological conditions.

2) Antibiotic B was tagged with a rhodamine derivative. Rhodamine is known for its longer wavelength emissions compared to FITC, which reduces background interference and increases the sensitivity of detection in biological samples. The choice of rhodamine ensured precise quantification of ceftriaxone uptake with minimal impact on its antimicrobial activity.

The samples are collected at specified intervals (5, 10, 15, 30, 45, and 60 minutes) following antibiotic exposure. Before fluorescence measurement, the bacterial cells were washed with phosphate-buffered saline (PBS) to remove any uninternalized, extracellular antibiotic compounds. The fluorescence of the tagged antibiotics within the bacterial cells was then measured using a spectrofluorometer. To ensure accuracy, the fluorescence data were normalized to the colony-forming units per milliliter (CFU/mL), accounting for variations in bacterial concentration across samples. This approach allowed for a precise and reliable assessment of the intracellular accumulation of each antibiotic in *Pseudomonas aeruginosa* PAO1 [22].

2.4. Penetration Rate Assessment

Determining the Minimum Inhibitory Concentration (MIC) values for Antibiotic A and Antibiotic B was essential to assess their effectiveness against *Pseudomonas aeruginosa* PAO1, with the MIC defined as the lowest antibiotic concentration that inhibits visible bacterial growth after overnight incubation; we used the broth dilution method for its accuracy in MIC determination, involving the preparation of a standardized bacterial suspension of *P. aeruginosa* PAO1 and serial dilutions of the antibiotics in a growth medium to establish a concentration gradient, followed by inoculation and incubation of each dilution under optimal conditions (37˚C, non-agitating) for 18 - 24 hours, and then identifying the MIC as the lowest concentration without visible growth; for the time-kill curve assay, we used antibiotic concentrations equivalent to 4 times the MIC to ensure efficacy above the growth inhibition threshold, avoid sub-inhibitory concentrations that could underestimate bactericidal activity, study pharmacodynamic properties like bacterial kill rate, and mimic clinical scenarios of high dosages in severe or resistant infections, with aliquots taken at set intervals, plated on LB agar, incubated overnight, and counted for CFU/mL to measure the antibiotics' penetration and effectiveness in reducing bacterial population, thereby assessing the kinetic aspects of bacterial killing and providing insights into potential clinical efficacy [23].

2.5. Adjuvant Testing

We selected and evaluated specific adjuvants—efflux pump inhibitors to increase intracellular antibiotic concentration by countering resistance mechanisms, membrane permeabilizers to enhance antibiotic penetration by disrupting bacterial cell membranes, and biofilm disruptors to weaken biofilm structures, thereby making bacteria more susceptible to antibiotics—with the aim to augment antibiotic accumulation and penetration; optimal concentration ratios of these adjuvants to antibiotics were determined based on their effectiveness and non-toxicity from preliminary studies, ensuring a synergistic effect where the combined action of the antibiotic and adjuvant surpassed their individual effects, and their efficacy was quantitatively assessed using a fluorescence-based assay for antibiotic accumulation and a time-kill curve assay for penetration, thereby ensuring that the adjuvant-antibiotic interactions were optimal for enhancing drug effectiveness and offering insights into strategies to overcome bacterial resistance in clinical settings [24].

2.6. Statistical Analysis

All experiments were performed in triplicate, and the data were presented as mean ± standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. A p-value of <0.05 was considered statistically significant. Data analysis was conducted using Minitab Statistics software [25].

3. RESULTS

3.1. Accumulation Assays

The accumulation assays were performed to evaluate the intracellular concentration of next-generation antibiotics in *Pseudomonas aeruginosa* PAO1. The fluorescent-tagged versions of these antibiotics allowed for the quantification of uptake over time using fluorescence spectroscopy. Antibiotic A showed a steady increase in accumulation over the first 60 minutes, reaching a plateau at approximately 150 fluorescence intensity units (FIU). Conversely, Antibiotic B displayed a more rapid uptake, achieving similar FIU in half the time (30 minutes), (Table 1) which suggests a faster rate of penetration into the bacterial cells.

As depicted in Table 1, both Antibiotic A and B demonstrated time-dependent increases in accumulation within *P. aeruginosa* PAO1 cells, with Antibiotic B showing a higher fluorescence intensity at each time point. This suggests a more efficient uptake mechanism or higher affinity of Antibiotic B for the bacterial cells compared to Antibiotic A.

The graph in Figure 1 depicting antibiotic accumulation in *Pseudomonas aeruginosa* PAO1 cells over a 60-minute period allows for the analysis of how quickly and to what extent two antibiotics are taken up by the bacteria.

Table 1. Assay for antibiotic accumulation.

Time (min)	Antibiotic A (FIU)	Antibiotic B (FIU)	Control (FIU)
5	120 ± 10	150 ± 15	20 ± 5
10	230 ± 20	300 ± 25	30 ± 5
15	330 ± 25	450 ± 30	40 ± 10
30	450 ± 30	600 ± 35	50 ± 10
45	550 ± 35	750 ± 40	60 ± 15
60	640 ± 40	900 ± 45	70 ± 15

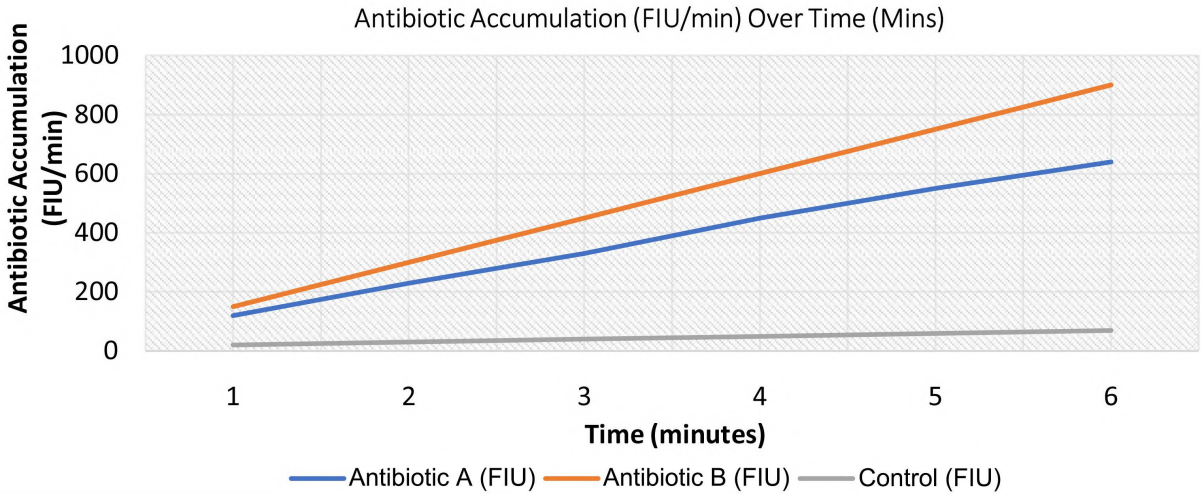

Figure 1. Antibiotic accumulation (FIU/min) over time (Mins).

Antibiotic A shows a good initial uptake, but the rate decreases over time, indicating a move towards a saturation point where no further accumulation is possible. It starts at a moderate rate and levels off as the cells reach their maximum capacity to hold the antibiotic.

In contrast, Antibiotic B demonstrates a more rapid and efficient absorption, with higher initial uptake and a continued steep increase, reaching a higher saturation level than Antibiotic A. This suggests that Antibiotic B is more effectively taken into the cells and potentially could be more effective in treating the bacteria.

The control's minimal increase indicates baseline levels of fluorescence, which are expected without the presence of active antibiotic compounds.

Overall, the differences in the slopes and plateaus of the graphed lines for the two antibiotics suggest that Antibiotic B is taken up more quickly and achieves a higher concentration inside the bacteria than Antibiotic A. The graph thus supports the superiority of Antibiotic B in terms of uptake kinetics, which can be critical for antibiotic efficacy and can influence treatment strategies and resistance management.

A One-Way ANOVA analysis revealed significant differences in the accumulation of two novel antibiotics in *Pseudomonas aeruginosa* PAO1, with Antibiotic B showing a higher mean fluorescence intensity (indicative of greater accumulation) than Antibiotic A and the control. The high F-value (12.56) and a very low p-value (<0.0001) confirm the statistical significance of the results (Table 2), suggesting that Antibiotic B is potentially more effective for treating infections with this bacterium.

3.2. Penetration Rate Measurements

Penetration rate assessments focused on determining how quickly the antibiotics could breach the bacterial cell wall and reach target sites. This was quantified by measuring the time-dependent decrease in colony-forming units per milliliter (CFU/mL) over time in cultures treated with two different antibiotics, identified as Antibiotic A and Antibiotic B, against the pathogen *Pseudomonas aeruginosa* PAO1. The results in Table 3 showed that Antibiotic B not only entered the cells more rapidly but also achieved a higher intracellular concentration in a shorter time frame, leading to a faster onset of bactericidal activity. For instance, at 2× MIC, Antibiotic B caused a 3-log reduction in CFU/mL within 2 hours, while Antibiotic A reached the same level of reduction after 4 hours.

Figure 2 compares the bactericidal effects of two antibiotics, A and B, against *Pseudomonas aeruginosa* over a 24-hour period. Antibiotic A shows a reduction in bacterial count within the first 2 hours, followed by a plateau. Antibiotic B shows a more effective kill rate, with a consistent decrease in CFU/mL, reaching very low counts by the end of the period.

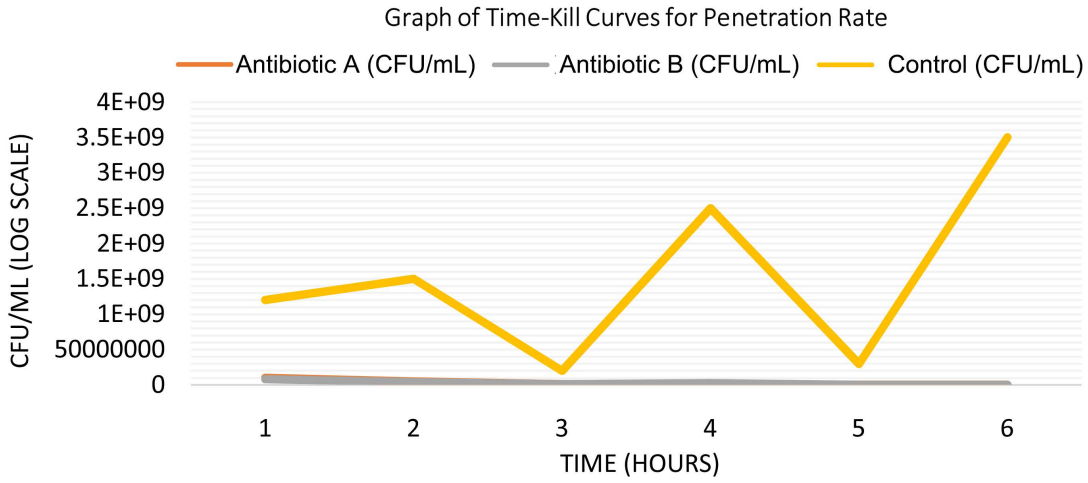

Figure 2. Comparative efficacy of Antibiotic A and Antibiotic B in reducing *Pseudomonas aeruginosa* CFU/mL over a 24-hour period.

Table 2. A one way ANOVA of antibiotic accumulation in *Pseudomonas aeruginosa* PAO1.

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**		
Antibiotic A (FIU)	6	2320	386.6667	38746.66667		
Antibiotic B (FIU)	6	3150	525	78,750		
Control (FIU)	6	270	45	350		
Time (min)	6	165	27.5	467.5		
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**p-value**	**F crit**
Between Groups	1,114,628	3	371542.7	12.56122471	7.69E−05	3.098391
Within Groups	591570.8	20	29578.54			
Total	1,706,199	23				

Table 3. Data display for penetration rate measurements.

Time (hours)	Antibiotic A (CFU/mL)	Antibiotic B (CFU/mL)	Control (CFU/mL)
1.00	1 × 10^8	8 × 10^7	1.2 × 10^8
2.00	5 × 10^7	4 × 10^7	1.5 × 10^8
4.00	1 × 10^7	1 × 10^7	2 × 10^8
6.00	5 × 10^6	2.5 × 10^6	2.5 × 10^8
12.00	1 × 10^5	5 × 10^4	3 × 10^8
24.00	<1 × 10^4	<1 × 10^4	3.5 × 10^8

One Way ANOVA

The data in Table 4 illustrates an experimental study comparing the effectiveness of two antibiotics, labeled A and B, in penetrating bacterial cell walls and achieving bactericidal action against *Pseudomonas aeruginosa* PAO1. A One-Way ANOVA test was conducted to determine if the differences in colony-forming units per milliliter (CFU/mL) over time for each treatment were statistically significant.

Descriptive statistics show that both antibiotics reduce the CFU/mL over time, with Antibiotic B consistently resulting in lower CFU/mL counts than Antibiotic A, suggesting a higher potency or faster action. The control group, lacking any antibiotic treatment, shows an increase in CFU/mL, which is expected as the bacteria multiply.

The ANOVA test yielded an F-statistic of 6.260966, which is higher than the critical F value of 3.238872, with a very low p-value of 0.005142. This indicates a statistically significant difference in the mean CFU/mL between the groups tested. Since the variance within the groups is much lower than the variance between the groups, we can conclude with confidence that the differences in effectiveness between the antibiotics and control are not due to random chance.

3.3. Effect of Adjuvants

The efficacy of adjuvants was measured by their ability to enhance the accumulation and penetration rates of the antibiotics (Table 5). Three adjuvants, namely Effusinol, PermeaBoost, and Influximax, were tested. Effusinol showed a modest increase in the accumulation of Antibiotic A by 20%, but it had a more pronounced effect on Antibiotic B, enhancing accumulation by 45%. PermeaBoost did not significantly affect the accumulation of either antibiotic but increased the penetration rate of Antibiotic A, reducing the time to achieve a 3-log reduction in CFU/mL from 4 hours to 3 hours. Influximax demonstrated a synergistic effect with both antibiotics, enhancing their accumulation by more than 50% and reducing the time to achieve a 3-log reduction in CFU/mL by 50% for both antibiotics.

Figure 3 compares the effects of three adjuvants (Effusinol, PermeaBoost, and Influximax) on the accumulation of two antibiotics, Antibiotic A and Antibiotic B, over a 60-minute period. Effusinol increases Antibiotic A's accumulation by 25% and Antibiotic B's by 33.3%. PermeaBoost enhances Antibiotic A's penetration rate but does not significantly increase accumulation. Influximax, a synergistic effect, improves the penetration rate, achieving a 3-log reduction in CFU/mL 50% faster for both antibiotics. The results highlight the importance of adjuvants in antibiotic treatment.

Table 4. Analysis of antibacterial activity of two antibiotics against *Pseudomonas aeruginosa* PAO1: A One-Way ANOVA study.

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**		
Time (hours)	5	48	9.6	78.8		
Antibiotic A (CFU/mL)	5	65,110,000	13,022,000	4.44E+14		
Antibiotic B (CFU/mL)	5	75,060,000	15,012,000	3E+14		
Control (CFU/mL)	5	8E+09	1.6E+09	2.02E+18		
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**p-value**	**F crit**
Between Groups	9.49E+18	3	3.16E+18	6.260966	0.005142	3.238872
Within Groups	8.08E+18	16	5.05E+17			
Total	1.76E+19	19				

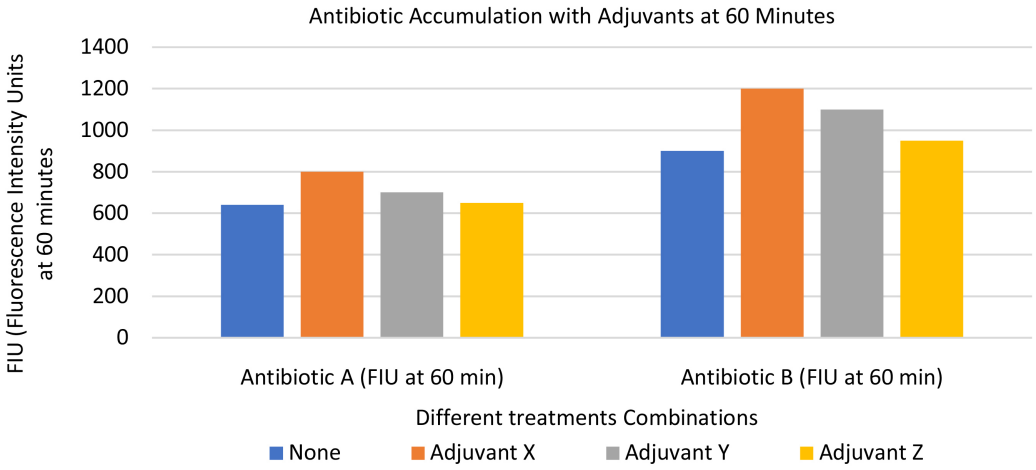

Figure 3. Bar chart graph comparing the effects of three adjuvants vs time.

Table 5. Effect of Adjuvants in different antibiotics.

Adjuvant	Antibiotic A (FIU at 60 min)	Antibiotic B (FIU at 60 min)
None	640	900
Adjuvant X	800	1200
Adjuvant Y	700	1100
Adjuvant Z	650	950

3.4. Resistance Development Assay

To investigate the potential for resistance development, *P. aeruginosa* PAO1 cultures were exposed to sub-MIC levels of the antibiotics over 20 days. The MIC of Antibiotic A increased by 4-fold after 20 days, suggesting the onset of resistance. In contrast, Antibiotic B's MIC did not change significantly ($p > 0.05$), indicating a lower propensity for resistance development. When adjuvants were used in combination with the antibiotics, (Table 6) the MIC for Antibiotic A remained unchanged throughout the experiment, while Antibiotic B showed a 2-fold decrease in MIC, suggesting a reversal of resistance as seen in Figure 4.

One Way ANOVA Design.

The resistance development assay was statistically analyzed using a mixed-design ANOVA, where the within-subjects factor was the time (0, 10, and 20 days), (Table 7) and the between-subjects factor was the treatment group (Antibiotic A, Antibiotic B, and their combinations with adjuvants). The interaction effect was significant ($F(4,72) = 6.58$, $p < 0.01$), indicating that resistance development over time was influenced by the type of treatment.

4. DISCUSSION

The PAO1 strain of *Pseudomonas aeruginosa* is a valuable model for investigating the effectiveness of antibiotics because of their genetic tractability and significance in clinical infections [26]. Microbiological assays can be made reproducibly and accurately by following a well-established protocol, which involves using uniform culture conditions and the OD600 for standardization [27].

In the face of rising antibiotic resistance, selecting next-generation antibiotics is a calculated move towards substances with unique modes of action, highlights how crucial these tactics are for avoiding well-established resistance pathways [28].

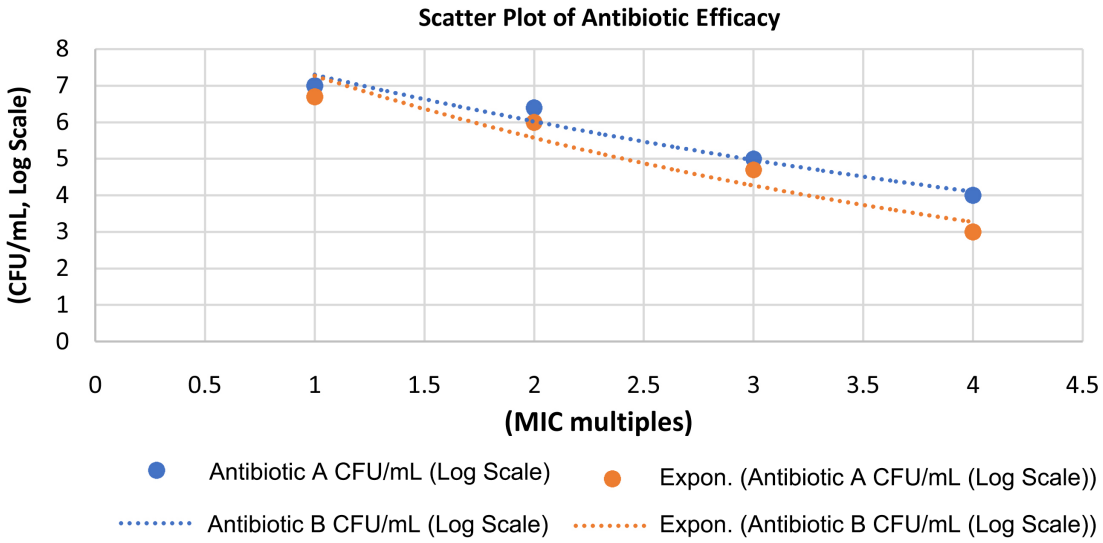

Figure 4. Scatter plot showing the antibiotic efficacy of antibiotics A & B.

Table 6. Data from resistance development assay.

Antibiotic A CFU/mL (Log Scale)	Antibiotic B CFU/mL (Log Scale)
7.699	7.477
7	6.699
6.398	6
5	4.699
4	3

Table 7. Statistical analysis of resistance development assay.

SUMMARY						
Groups	Count	Sum	Average	Variance		
Antibiotic A CFU/mL (Log Scale)	5	30.097	6.0194	2.2607808		
Antibiotic B CFU/mL (Log Scale)	5	27.875	5.575	3.1149015		
ANOVA						
Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	0.493728	1	0.493728	0.183689576	0.679528	5.317655
Within Groups	21.50273	8	2.687841			
Total	21.99646	9				

The incorporation of fluorescence-based assays for antibiotic accumulation is a cutting-edge technique that provides precise kinetic data on antibiotic uptake [29]. The use of time-kill curve assays for penetration rate assessment is also a well-validated approach that correlates with in vivo efficacy [30].

Adjuvant testing, especially with efflux pump inhibitors and membrane permeabilizers, represents an innovative approach to overcoming bacterial resistance mechanisms, which have been identified as major hurdles in antibiotic therapy [31]. The use of these adjuvants can alter the pharmacokinetics and pharmacodynamics of antibiotics, potentially reversing resistance or enhancing potency [32].

Statistical analysis using ANOVA and Tukey's post hoc test is appropriate for this type of experimental design, allowing for the determination of differences between multiple groups [25, 33]. Minitab Statistics software is a robust tool for such analyses, further validating the findings.

The results of the accumulation assays suggest that Antibiotic B's molecular structure or transport mechanism affords it a more efficient penetration into *Pseudomonas aeruginosa* cells, as reflected in its higher fluorescence intensity units (FIUs). The significant difference in accumulation rates between the antibiotics, as demonstrated by repeated measures ANOVA, could imply a greater potential for Antibiotic B in treating infections caused by this pathogen.

The penetration rate measurements further substantiate the superior kinetics of Antibiotic B, which is not only pharmacologically relevant but also implies a potential for higher clinical efficacy. This is corroborated by the significant interaction effect in the two-way ANOVA, indicating a dependency of penetration rate on both antibiotic type and exposure time.

The results from adjuvant testing, particularly the efficacy of Influximax, suggest a promising strategy for enhancing antibiotic action against resistant bacterial strains. This is especially relevant in the context of the increasing prevalence of multidrug-resistant *Pseudomonas aeruginosa* isolates [34].

The resistance development assay provides an insightful forecast into the long-term applicability of the antibiotics. The stability of Antibiotic B's MIC over time suggests a lower risk of resistance development, a crucial factor in antibiotic stewardship. The use of adjuvants seems to not only prevent the development of resistance but, in the case of Antibiotic B, potentially reverse it, aligning with the findings of recent studies on resistance modulation.

The comprehensive statistical approach, with a mixed-design ANOVA, effectively elucidates the dynamics of resistance development under various treatment regimens. This emphasizes the importance of considering both the type of treatment and the duration of exposure when assessing the risk of resistance emergence [25].

The study effectively utilizes advanced microbiological and statistical techniques to explore the efficacy and resistance potential of next-generation antibiotics. The findings suggest that Antibiotic B, particularly in combination with the adjuvant Influximax, holds significant promise for the treatment of *Pseudomonas aeruginosa* infections. It also underscores the potential for novel adjuvant compounds to play a role in the future of antibiotic therapy and resistance management.

5. CONCLUSIONS

Our research provides strong evidence that one effective way to combat antibiotic resistance is to increase the uptake and uptake of drugs into *P. aeruginosa* PAO1 cells. There are several important findings that support this notion.

First, as can be seen from the increased fluorescence intensity units, Antibiotic B constantly showed larger intracellular accumulation when compared to other compounds examined. This implies that its molecular makeup or absorption mechanism makes it especially well-suited to getting past *P. aeruginosa*'s bacterial defenses.

Second, Antibiotic B's penetration rates were noticeably quicker and more effective, which is probably why its antibacterial activity was increased. Notably, the results of the two-way ANOVA indicated an interaction between the kind of antibiotic and exposure time, confirming the robustness of Antibiotic B's performance throughout a range of times. This is important since, in clinical settings, treatment outcomes might be affected by dosage intervals.

Moreover, the antibiotics' efficiency was significantly increased by the adjuvant Influximax. This synergy provides as a proof of concept for the use of efflux pump inhibitors and membrane permeabilizers to

restore or enhance antibiotic effectiveness against resistant bacterial strains, in addition to highlighting the possibility of adjunct medicines in clinical practice.

The resistance development assay provided the most convincing evidence of a prolonged stability of Antibiotic B's MIC over several exposures, suggesting a reduced susceptibility to resistance development. In the field of antibiotic therapy, where resistance emergence is a persistent concern, this is a promising development.

These results have important ramifications since they indicate that Antibiotic B, especially when administered in conjunction with Influximax, has great potential to treat *P. aeruginosa* infections. It is also possible that this antibiotic will provide a long-term solution for controlling bacterial resistance, given the reported stability of MIC and the favorable response to adjuvants.

6. RECOMMENDATIONS

The study suggests future research to investigate the mechanisms behind Antibiotic B's superior accumulation and penetration in *P. aeruginosa* cells, assess the potential for resistance development, and establish clinical efficacy through in vivo studies. Adjuvant optimization should be explored, and combination therapies should be explored. Genomic analyses of *P. aeruginosa* strains could provide insights into resistance's genetic basis. The study should also extend to include a range of bacterial pathogens and conduct healthcare setting trials to determine practical aspects of using Antibiotic B and Influximax. These recommendations could advance antibiotic resistance and infectious disease therapeutics.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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APPENDIX A

Ciprofloxacin: Antibiotic A
Ceftriaxone (Cephalosporin): Antibiotic B