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Human Beta-Defensin-3 and Di-Aminoimidazole as Antimicrobial Adjuvants Against Multidrug-Resistant Acinetobacter baumannii

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ABSTRACT

Background: Multidrug-resistant (MDR) Acinetobacter baumannii is a major cause of hospital-acquired infections with high morbidity and mortality. Resistance has quickly grown, making it harder to find effective treatments. This has led to the need for new approaches, like antimicrobial adjuvants that make existing antibiotics work better.

Objective: This study assessed the efficacy of human beta-defensin-3 (hBD-3) and di-aminoimidazole (2-AI) as antimicrobial adjuvants in conjunction with conventional antibiotics against multidrug-resistant A. baumannii.

Methods: We evaluated three MDR clinical isolates and one reference strain (ATCC19606). Used broth microdilution to find the minimum inhibitory concentrations (MICs) of hBD-3, 2-AI, and four antibiotics: ampicillin-sulbactam, imipenem, ceftazidime, and ciprofloxacin. A sub-MIC quantity of 50 μg/mL of hBD-3 and 2-AI was employed in combinatorial experiments. Experiments three times and used the paired t-test and Wilcoxon signed-rank test to look at the results.

Results: A notable decrease in MIC occurred when ampicillin-sulbactam was administered in conjunction with either hBD-3 or 2-AI across all isolates. There was also a big improvement with imipenem+ hBD-3, however imipenem + 2-AI had no impact. No substantial synergy was seen for combinations of ceftazidime or ciprofloxacin with either adjuvant.

Conclusion: hBD-3 and 2-AI showed potential, as antimicrobial adjuvants, especially; when combined with β-lactam antibiotics. However, due to the limited number of isolates examined, these findings must be considered preliminary, and further studies with larger sample sizes are required.

Keywords: Acinetobacter baumannii; multidrug resistance; antimicrobial adjuvants; human beta-defensin-3; di-aminoimidazole.

1. Introduction

Acinetobacter baumannii has become one of the most troublesome nosocomial diseases, because it can ability quickly, become resistance to several types of antibiotics. Infections produced by multidrug-resistant (MDR) A. baumannii are linked to high rates of illness and death, especially in critically sick patients, with death rates ranging from 20% to 60% [1-3]. The organism is often isolated from respiratory secretions, and burn wound samples, indicating its capacity to endure in hospital settings and colonize exposed tissues [4].

The growing resistance to last-line antibiotics, such as carbapenems, has greatly reduced the treatment choices for A. Baumannii infections [5,6]. This resistance is facilitated by various processes, including enzymatic degradation of antibiotics, changes in target genes, overexpression of efflux pumps, and diminished outer membrane permeability [7,5]. As a result, treatment failure has become more likely in infections caused by MDR A. baumannii [2].

A number of measures have been suggested to deal antimicrobial resistance, such as antibiotic stewardship programs, the creation of new antimicrobial drugs, and combination antibiotic therapy [2]. However, these approaches face major limitations. The discovery of new antibiotics is costly and time-consuming, and resistance frequently emerges shortly after their introduction [8]. Therfore, alternative strategies aimed enhancing, the efficacy of existing antibiotics without exerting strong selective pressure on, bacteria have gained increasing attention in recent years [9,10].

Antimicrobial adjuvants, represent a promising approach to overcome antibiotic resistance by restoring or enhancing the activity of existing antimicrobial agents [10,11]. Human beta-defensin-3 (hBD-3) is a cationic antimicrobial peptide synthesized, by epithelial cells, demonstrating extensive antimicrobial efficacy by compromising bacterial membrane integrity and obstructing vital biological functions 12,9. Furthermore; hBD-3 has demonstrated the ability to augment bacterial susceptibility to standard antibiotics [13].

Antimicrobial peptides; such as hBD-3 can also disrupt bacterial membranes and facilitate antibiotic penetration, thereby, increasing the capability of multidrug-resistant bacteria to conventional antibiotics [14]. Di-amino-imidazole (2-AI) compounds have garnered significant attention for their capacity to inhibit biofilm formation, and alter resistance mechanisms in Gram-negative bacteria [10,15]. Previous studies has shown that 2-AI derivatives can synergistically enhance the antibacterial activity of conventional antibiotics against multidrug resistant organisms, including Acinetobacter species [11,15]. This study aims to investigate the efficacy of human beta-defensin-3 and di-aminoimidazole as antimicrobial adjuvants in combination with specific antibiotics against multidrug resistant Acinetobacter baumannii isolates [16].

2. Materials and Methods

2.1. Bacterial isolates

A total of 100 clinical isolates were initially collected and identified as *Acinetobacter baumannii*. From these isolates, three multidrug-resistant (MDR) *A. baumannii* clinical isolates were selected based on their high resistance profiles. Identification and antimicrobial susceptibility profiles were previously determined using the the VITEK-2 automated system. In addition, one standard reference strain of *Acinetobacter baumannii* (ATCC 19606) was included in the study as a control.

2.2. Minimum Inhibitory Concentration (MIC) Determintion

The bacterial inoculum for the three selected MDR clinical isolates and the ATCC reference strain was prepared from ten single colonies grown for 24 hours on Mueller-Hinton agar (MHA). The colonies were suspended in 2mL of sterile distilled water. The suspension was adjusted spectrophotometrically at 600nm (OD_{600}) using a BioTek 800ST plate reader (BioTek, USA) to a final OD_{600} of 0.236, corresponding to a McFarland standard of 0.67.

The stock suspension was then diluted 100-fold in Mueller-Hinton broth (MHB) to achieve a final working inoculum of 1×10^6 CFU/mL. Avolume of 50 µL of this inoculum was used to inoculate each well,except for the blank (sterility control) wells. The MICs of the tested antibiotics (ampicillin-sulbactam,

imipenem, ceftazidime, and ciprofloxacin), as well as the MICs of human beta-defensin-3 (hBD-3) and di-aminoimidazole (2-AI), were determined using the broth microdilution method as described by Ardalani et al.[16]. All assays were performed in triplicate and included positive (growth control) and negative (broth control) wells. The assay was conducted in 96-well microtiter plates with a final volume of 225 µL per well.The tested compounds were prepared to achieve the following final concentration ranges: ampicillin-sulbactam (11.0-2.0µg/mL), imipenem (9,3-1.7 µg/mL), ceftazidime (50.0-2.3 µg/mL), and ciprofloxacin (16.0-0.7 µg/mL).Following overnight incubation, bacterial growth was assessed by measuring optical density at 600nm. Cell viability was calculated by comparing the optical density of treated wells with that of the untreated control wells. The MIC was defined as the lowest concentration of the tested compound that completely inhibited visible bacterial growth.

Statistical analysis

All experiments were complete in triplicate, and results were expressed as mean± SD. Differences between treatments were analyzed using the paired t-test for normally distributed data, and the Wilcoxon signed-rank test for non-normally distributed data. To choose the right test, data distribution was assessed before statistical analysis. A p- value<0.05 was considered statistically significant.

3. Results

3.1 MIC Determination of hBD-3 and 2-AI

The minimum inhibitory concentrations (MICs) of human beta-defensin-3 (hBD-3) and di-aminoimidazole (2-AI) against the selected multidrug-resistant (MDR) *Acinetobacter baumannii* isolates and the ATCC reference strain were determined prior to the combination assays. The MIC values of 2-AI are presented in Table 1, and the corresponding MIC determination is illustrated in Figure 1.

Similarly, the MIC values of hBD-3 are shown in Table 2, with graphical representation provided in Figure 2. Based on these results, a sub-MIC concentration of 50 µg/mL was selected for subsequent combination experments.

Table 1. MIC values of di-aminoimidazole (2-AI) against *Acinetobacter baumannii* isolates.

St1	St2	St3	Std
150	300	300	300

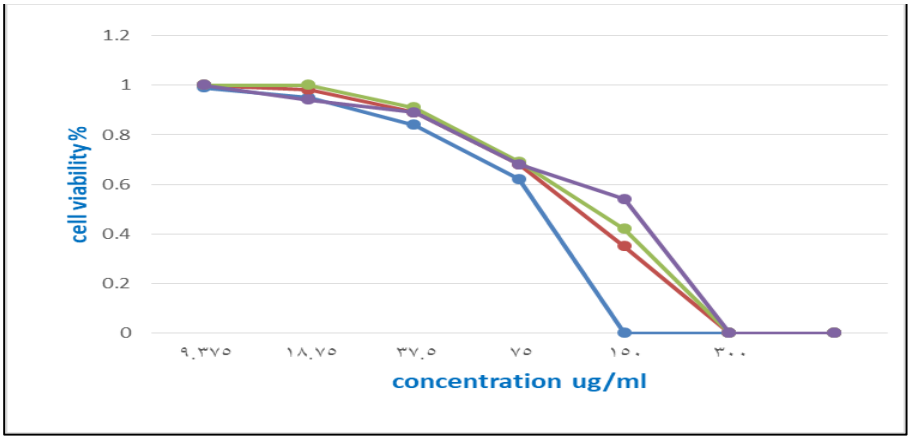

Figure 1. Determination of the MIC of di-aminoimidazole (2-AI) against *Acinetobacter baumannii* isolates

Table 2. MIC values of human beta-defensin-3 (hBD-3) against *Acinetobacter baumannii* isolates

hBD-3	St1	St2	St3	Std
	>300	>300	>300	>300

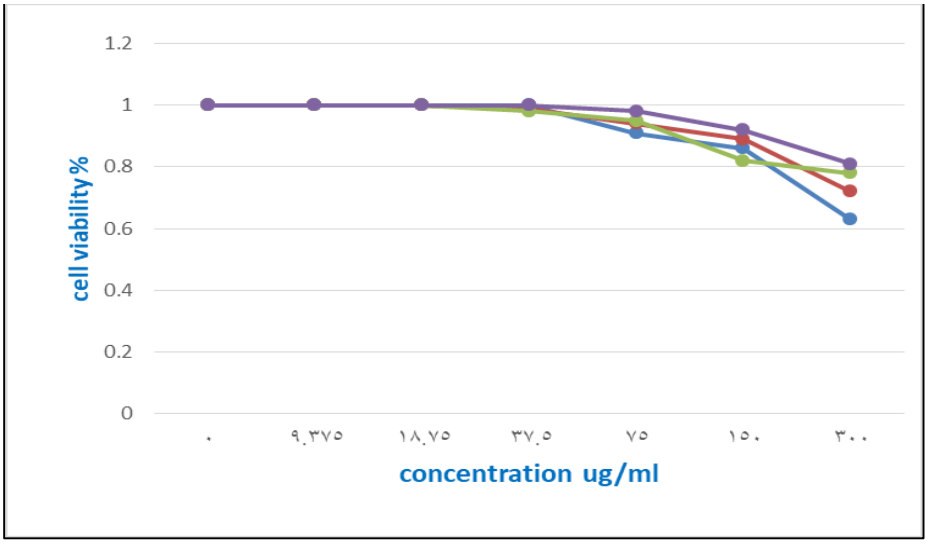

Figure 2. Determination of the MIC of human beta-defensin-3 (hBD-3) against *Acinetobacter baumannii* isolates.

3.2 Effect of ampicillin-sulbactam combined with hBD-3 and 2-AI

The combination of ampicillin-sulbactam with hBD-3 resulted in a significant reduction in MIC values across all tested MDR *A.baumannii* isolates compared with ampicillin-sulbactam alone. The MIC values for this combination are summarized in Table 3, while the corresponding growth inhibition profiles are illustrated in Figure 3. Similary, combining ampicillin-sulbactam with 2-AI resulted in a reduction in MIC values against the tested isolates. However, some of these reductions were modest and not statistically significant (P> 0.05). The MIC values of this combination are presented in Table 4, and the growth inhibition curves are shown in Figure 4.

Table 3. MIC values of ampicillin-sulbactam alone and in combination with human bête-defensin-3

MIC (µg/ml)	St1	St2	St3	Atcc	Wilcoxon Signed Ranks Test (P value)	Paired T test (p value)
Ampicillin-sulbactam	11	11	11	8.3	0.05	0.003
Ampicillin-sulbactam +hBD-3	4.6	4.6	4.6	4.6		

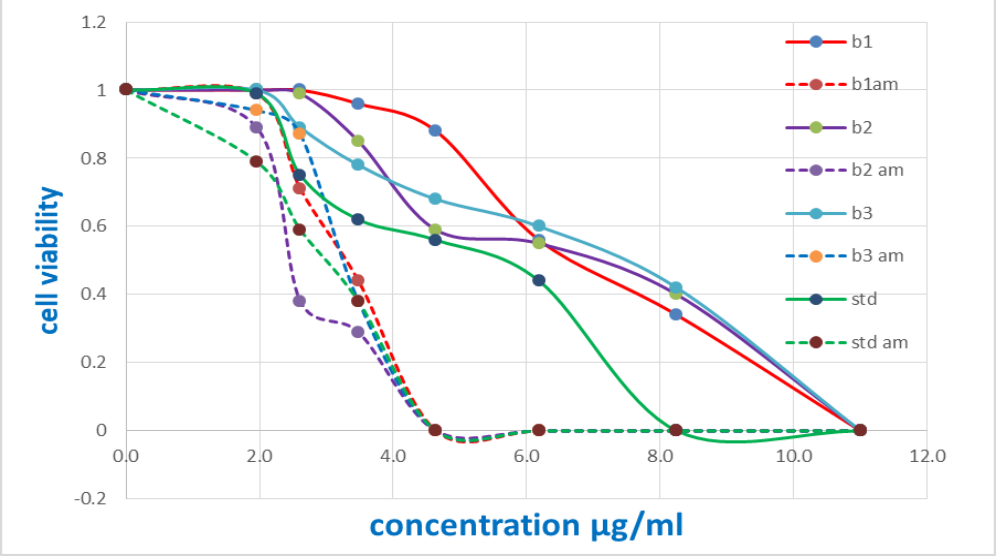

Figure 3. Growth inhibition of *A.baumannii* treated with ampicillin-sulbactam± hBD-3.

Table 4. MIC values of ampicillin-sulbactam alone and in combination with di-aminoimidazole (2-AI) .

MIC (µg/ml)	St1	St2	St3	Atcc	Wilcoxon Signed Ranks Test (P value)	Paired T test (p value)
Ampicillim – sulbactam	11	11	11	8.3	0.066	0.009
Ampicillin – sulbactam + 2-Aminoimidazole	4.6	2.6	2.6	4.6		

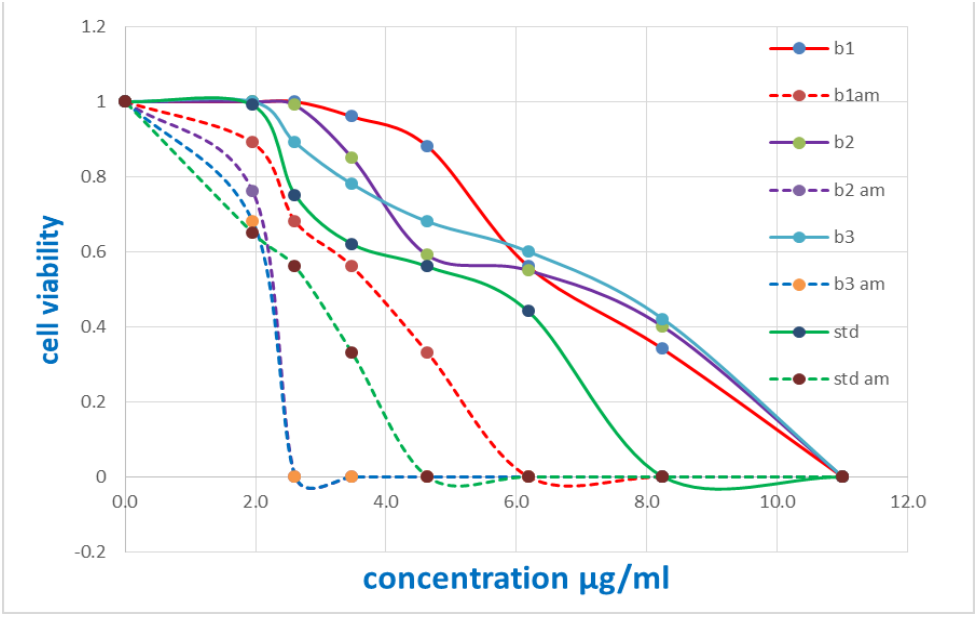

Figure 4. Growth inhibition of *Acinetobacter baumannii* treated with ampicillin-sulbactam± 2-AI

3.3 Effect of imipenem combined with hBD-3 and 2-AI

A significant enhancement of antibacterial activity was observed when imipenem was combined with hBD-3 against the tested MDR *A. baumannii* isolates. The MIC values obtained for this combination are presented in Table 5, and bacterial growth inhibition is illustrated in Figure 5.z\

In contrast, the combination of imipenem with 2-AI did not result in significant change in MIC values compared with imipenemnalone. The MIC values and growth response for this combination are shown in Table 6 and Figure 6, respectively.

Table 5. MIC values of imipenem alone and in combination with human bête-defensin-3 (hBD-3).

MIC (µg/ml)	St1	St2	St3	Atcc	Wilcoxon Signed Ranks Test (P value)	Paired T test (p value)
Imipenem	9.3	9.3	9.3	3.9	0.043	0.035
Imipenem +hBD-3	4.6	4.3	4.1	2.0		

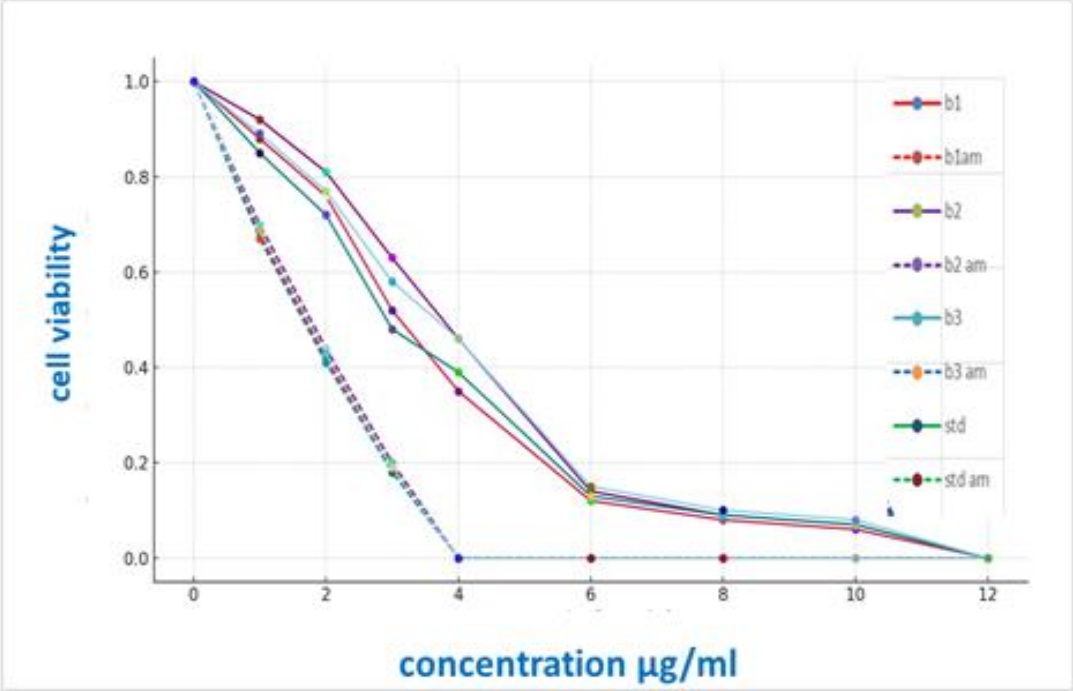

Figure 5. Growth inhibition of Acinetobacter baumannii treated with imipenem± hBD-3.

Table 6. MIC values of imipenem alone and in combination with di-aminoimidazole (2-AI)

MIC (µg/ml)	St1	St2	St3	Atcc	Wilcoxon Signed Ranks Test (P value)	Paired T test (p value)
Imipenem	9.3	9.3	9.3	3.9	1	**1**
Imipenem +2-Aminoimidazole	9.3	9.3	9.3	3.9		

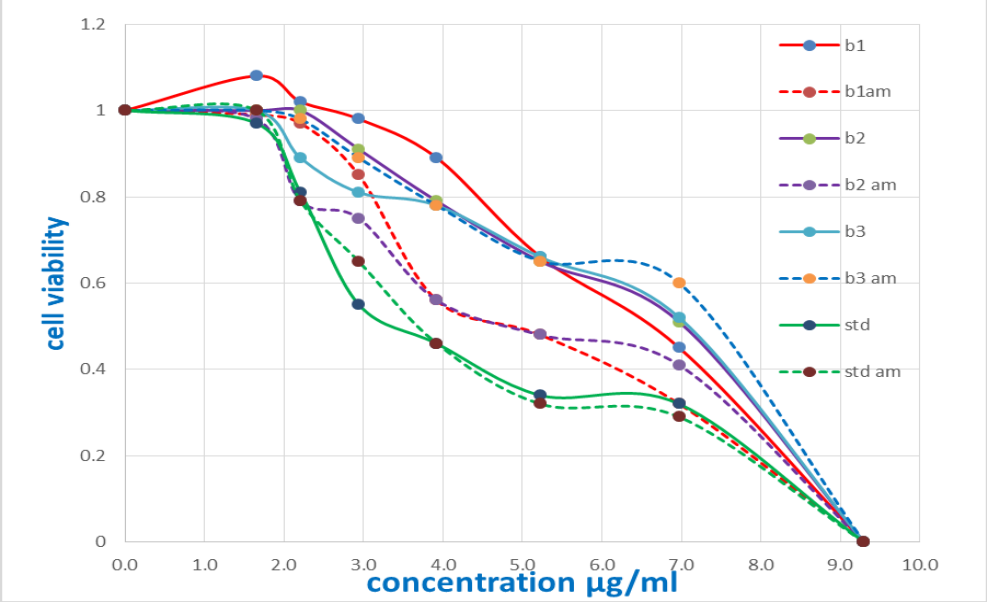

Figure 6. Growth response of Acinetobacter baumannii treated with imipenem ± 2-AI.

3.4 Effect of ceftazidime and ciprofloxacin combinations

NO significant inhibitory effects were observed when ceftazidime was combined with either hBD-3 0r 2-AI. The MIC values for these combinations are presented in Tables 7 and 8, and the corresponding bacterial growth responses are illustrated in Figures 7 and 8. Similaly, combining ciprofloxacin with hBD-3 or 2-AI did not result in a significant enhancement of antibacterial activity against the tested isolates. The MIC values for ciprofloxacin combinations are summarized in Tables 9 and 10, with growth responses illustrated in Figures 9 and 10.

Table 7. MIC values of ceftazidime alone and in combination with human beta-defensin-3 (hBD

MIC (µg/ml)	St1	St2	St3	atcc	Wilcoxon Signed Ranks Test (P value)	Paired T test (p value)
ceftazidime	50	3.9	50	50	1	1
Ceftazidime + hBD-3	50	3.9	50	50		

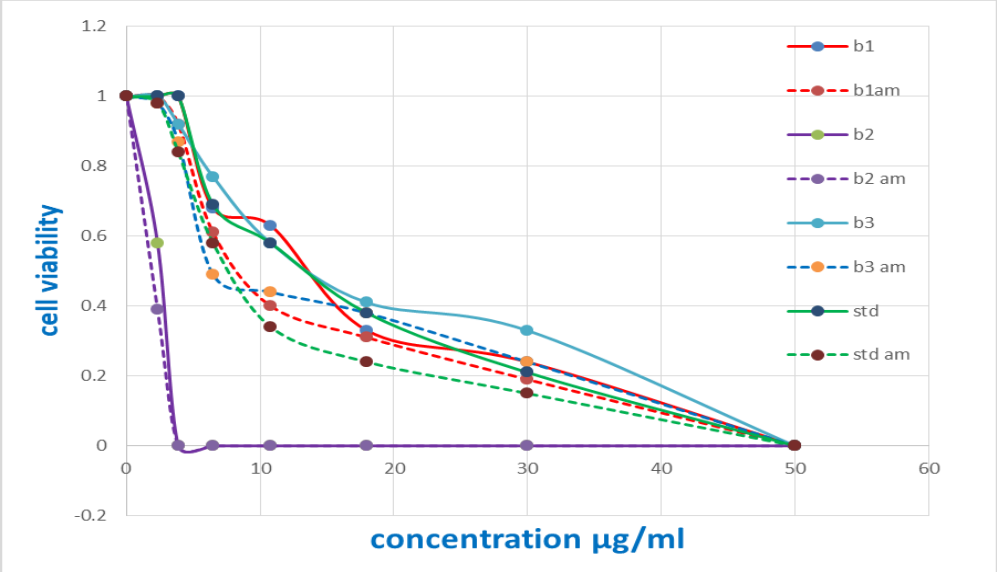

Figure 7. Growth response of Acinetobacter baumannii treated with ceftazidime ± hBD-3.

Table 8. MIC values of ceftazidime alone and in combination with di-aminoimidazole (2-AI).

MIC(µg/ml)	St1	St2	St3	atcc	Wilcoxon Signed Ranks Test (P value)	Paired T test (p value)
Ceftazidime	50	3.9	50	50	0.10	0.107
ceftazidime + 2-Aminoimidazole	30	3.9	30	3.9		

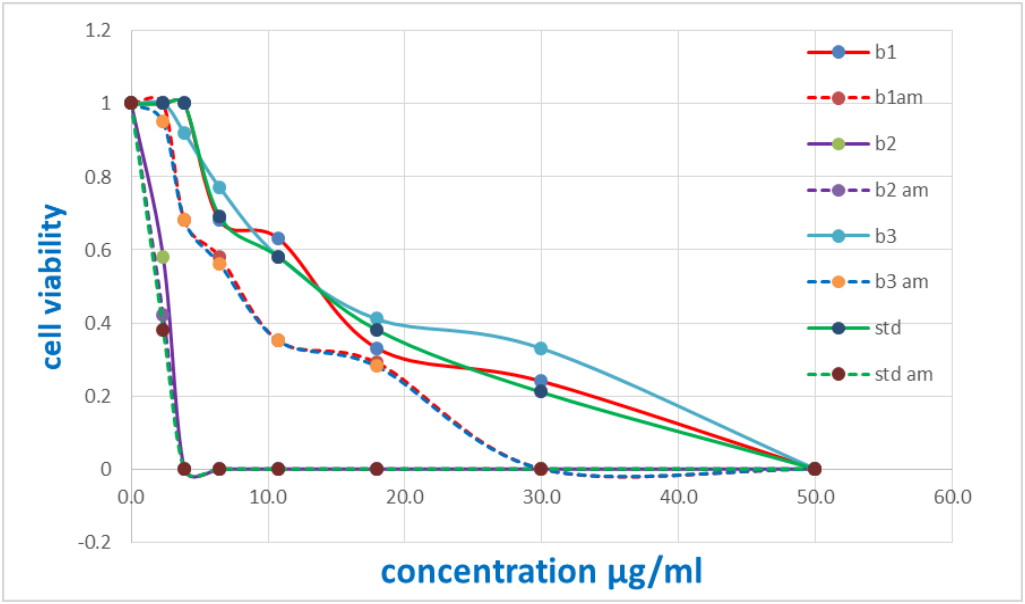

Figure 8. Growth response of *Acinetobacter baumannii* treated with ceftazidime ± 20AI

Table 9. MIC values of ciprofloxacin alone and in combination with human beta-defensin-3 (hBD-3)

MIC(µg/ml)	St1	St2	St3	atcc	Wilcoxon Signed Ranks Test (P value)	Paired T test (p value)
Ciprofloxacin	2.1	16	2.1	1.2	0.18	0.3
Ciprofloxacin + hBD-3	1.2	5.8	2.1	1.2		

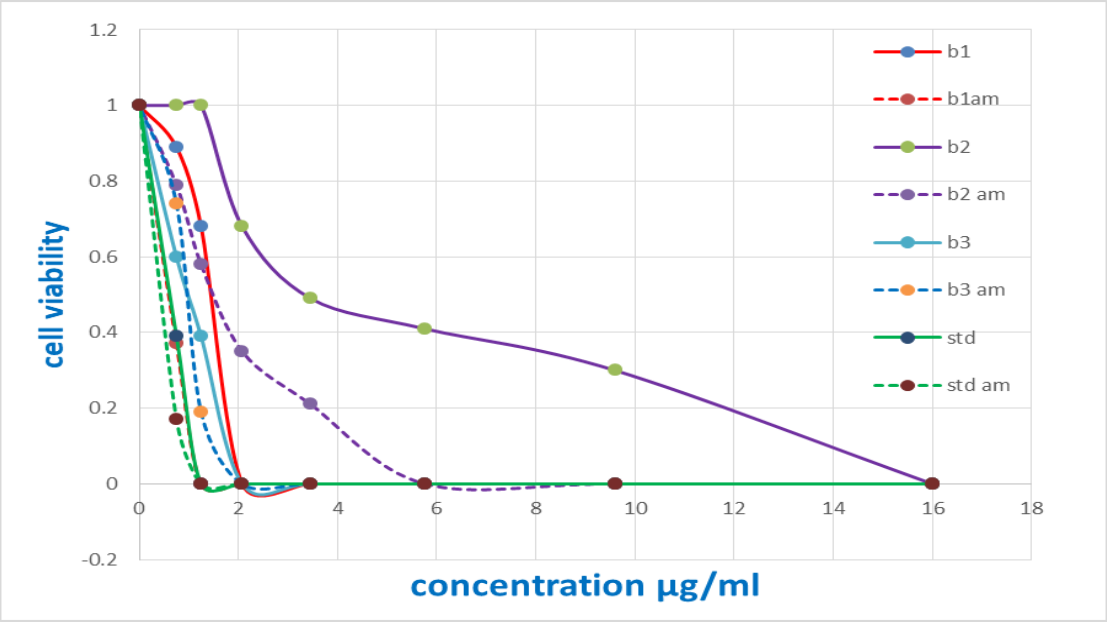

Figure 9. Growth response of *Acinetobacter baumannii* treated with ciprofloxacin ± hBD-3.

Table 10. MIC values of ciprofloxacin alone and in combination with di-aminoimidazole (2-AI).

MIC(µg/ml)	St1	St2	St3	atcc	Wilcoxon Signed Ranks Test (P value)	Paired T test (p value)
Ciprofloxacin	2.1	16	2.1	1.2	0.18	0.3
Ciprofloxacin + 2-Aminoimidazole	1.2	2.1	2.1	1.2		

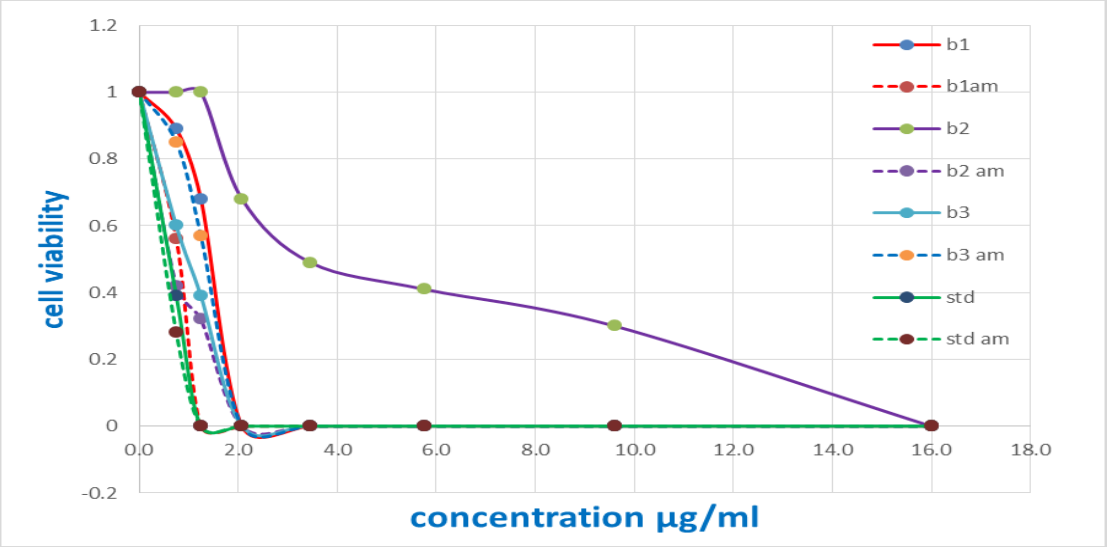

Figure 10. Growth response of *Acinetobacter baumannii* treated with ciprofloxacin ± 2-AI.

4. Discussion

hBD-3 is known to break down the integrity of bacterial membranes and make them more permeable, which may let antibiotics get into the bacterial cell more easily.

The present study evaluated the potential of human beta-defensin-3 (hBD-3) and di-aminoimidazole (2-AI) as antimicrobial adjuvants in combination with selected antibiotics against multidrug-resistant (MDR) *Acinetobacter baumannii* . The results show that the effectiveness of these adjuvants dependent a lot on the antibiotic employed, which shows how complicated the interactions between antibiotics and adjuvant can be.

The most consistent and significant enhancement of antibacterial activity was observed when hBD-3 or 2-AI was combined with ampicillin-sulbactam. This enhancement may be ascribed to the synergistic mechanisms of action between β-lactam antibiotics and the evaluated adjuvants. hBD-3 is known to break down the integrity of bacterial membranes and make them more permeable, which may let antibiotics get into the bacterial cell more easily. In the same way, 2-AI compounds has been reported to interfere with biofilm formation and resistance-associated pathways, potentially increasing bacterial susceptibility to β-lactam antibiotics.

In contrast, the combination of imipenem with hBD-3 showed a significant effect, wheras the combination of imipenem with 2-AI did not produce a comparable improvement. This difference may reflect the distinct chemical properties and stability of imipenem, as well as potential variations in how each adjuvant interacts with the bacterial cell envelope and resistance mechanisms.

No significant enhancement was observed when either hBD-3 or 2-AI was combined with ceftazidime or ciprofloxacin. These findings suggest that not all antibiotic-adjuvant combinations are beneficial and that the success of adjuvant therapy is strongly influenced by the specific antibiotic class and its mechanism of action. Furthermore, although reductions in MIC values were observed in some combinations, several of these changes were modest and did not reach statistical significance. This may be attributed to the limited number of isolates examined in the present study.

The lack of effect in these combinations may be related to pre-existing resistance mechanisms in the lack of effect in these combination may be related to pre-existing resistance mechanisms in the tested isolates that are not effectively targeted by hBD-3 or 2-AI. Overall, the results support the concept that antimicrobial adjuvants can restore or enhance the activity of certain antibiotics against MDR *A.baumannii*, particularly β-lactam-basad regimens such as ampicillin-sulbactam. However, further studies are needed to clarify the precise molecular mechanisms underlying these interactions and to evaluate their clinical applicability.

5. Conclusion

The findings suggest that human beta-defensin-3 (hBD-3) and 2-aminoimidazole may enhance the activity of certain β-lactam antibiotics against multidrug-resistant *Acinetobacter baumannii* in vitro. However, because of the limited number of isolates and the absence of formal synergy analysis, these results should be considered preliminary. Further studies using larger sample sizes, formal synergy assays, and in vivo models are required to confirm these observations.

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