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In-Vitro Synergistic Activity of Nortriptyline in Combination with Vancomycin Against a Clinical Vancomycin-Intermediate Staphylococcus aureus (VISA): A Proof-of-Concept Study

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Abstract

Background: *Staphylococcus aureus* is an important pathogen responsible for a wide range of community-and hospital- acquired infections. The increasing prevalence of strains with reduced vancomycin susceptibility has limited therapeutic options. It underscores the need to identify novel antimicrobial combinations.

Objectives: This proof-of-concept study aimed to evaluate the in vitro antibacterial activity of Nortriptyline and to investigate its interaction with vancomycin against a clinical *vancomycin- intermediate Staphylococcus aureus (VISA)* isolate.

Methods: In this in vitro experimental study, a single clinical *VISA* isolate was evaluated. The minimum inhibitory concentrations (MICs) of Nortriptyline and vancomycin were determined by broth microdilution. Drug interactions were assessed using the checkerboard assay, and interactions were interpreted based on the fractional inhibitory concentration index (FICI).

Results: The MICs of vancomycin and Nortriptyline alone were 7.81 µg/ml and 1000 µg/ml, respectively. When combined, the MICs of both agents decreased fourfold to 1.95 µg/ml and 250µg/ml respectively. The calculated FICI was 0.5, indicating synergistic activity according to the predefined interpretation criteria.

Conclusion: Nortriptyline exhibited in vitro synergistic activity in combination with vancomycin against a clinical VISA isolate. These preliminary findings provide a rationale for further investigation of this combination in larger collections of clinical isolates and complementary experimental models.

Keywords: Drug repurposing, Antimicrobial resistance, Nortriptyline. *Staphylococcus aureus*, vancomycin.

1. Introduction

Bacterial and fungal infections remain important challenges in both medicine and dentistry. Among bacterial pathogens, Staphylococcus aureus is one of the most common causes of skin, soft tissue, and mucosal infections, particularly in the oral cavity(1, 2). Owing to its diverse virulence factors, including toxins, adhesins, and capsular components, S. aureus can cause a wide range of clinical infections. In addition, interactions between S. aureus and fungal pathogens, particularly Candida albicans, may contribute to mixed oral infections, including angular cheilitis, median rhomboid glossitis, and denture stomatitis (2, 3). In recent decades, the global increase in antimicrobial resistance has created significant challenges in the management of bacterial infections. The emergence and dissemination of methicillin-resistant Staphylococcus aureus (MRSA) strains have substantially limited available therapeutic options. Vancomycin has long been considered a cornerstone treatment for serious MRSA infections; however, the emergence of strains with reduced vancomycin susceptibility, including vancomycin-intermediate Staphylococcus aureus (VISA) and vancomycin-resistant Staphylococcus aureus (VRSA), represents an increasing therapeutic concern (4-7).

Faced with the global challenge of antimicrobial resistance and the limited

development of new antibiotics, alternative strategies, such as antimicrobial combination therapy and drug repurposing, have attracted considerable attention. Drug repurposing involves evaluating previously approved non-antibiotic drugs as potential antimicrobial agents or antibiotic adjuvants. This approach may provide opportunities to enhance the activity of existing antibiotics; however, in vitro findings require further validation before clinical application (8, 9).

Scientific evidence has demonstrated that some non-antibiotic drugs, including tricyclic antidepressants (TCAs), possess antimicrobial properties. Previous studies have reported antibacterial activity of TCAs against Gram-positive bacteria, particularly S. aureus. These compounds have been suggested to affect bacterial viability through multiple mechanisms, including alterations in membrane integrity, induction of oxidative stress, and modulation of bacterial efflux systems; however, the precise mechanisms underlying their activity remain incompletely understood (9-12). Nortriptyline is a widely used tricyclic antidepressant with established pharmacological properties and clinical applications. Beyond their neurological effects, Nortriptyline and related TCAs have attracted interest for their potential antimicrobial activity. Previous investigations have reported inhibitory effects of Nortriptyline against microbial pathogens, particularly fungal species, and synergistic interactions with antifungal agents such as amphotericin B. Furthermore, recent studies have demonstrated antibacterial activity of Nortriptyline against S. aureus, either alone or in combination with conventional antibacterial agents, supporting its potential as an antimicrobial adjuvant (13).

Despite these findings, limited information is available on Nortriptyline's ability to enhance activity against S. aureus strains with reduced vancomycin susceptibility, particularly VISA isolates. However, the interaction between Nortriptyline and vancomycin against VISA isolates has not been adequately investigated. Therefore, this study aimed to evaluate the antibacterial activity of Nortriptyline alone and its interaction with vancomycin against a single clinical VISA isolate using the checkerboard method and the fractional inhibitory concentration index (FICI).

2. Methods

2.1 Study Design and Ethical Considerations

This basic experimental study was an in vitro investigation conducted in the microbiology laboratory of Arak University of Medical Sciences. The university's ethics committee approved the study protocol under code "IR.ARAKMU.REC.1403.037".

2.2. Bacterial Strain and Culture Conditions

A single clinical isolate of Staphylococcus aureus with reduced vancomycin susceptibility was obtained from the Department of Microbiology and Immunology's bacterial biobank at Arak University of Medical Sciences. The isolate was initially identified as S. aureus using conventional biochemical tests. It was confirmed to exhibit a vancomycin MIC of 4-8 µg/ml according to CLSI guidelines before this study(14). The bacterium was cultured on Mueller-Hinton Agar (MHA) and incubated at 37ºC for 24 hours. For subsequent experiments, several colonies were transferred to Mueller- Hinton Broth (MHB) and incubated at 37ºC for 18-24 hours to reach the mid-logarithmic growth phase.

2.3. Preparation of Inoculum

The bacterial suspension was adjusted to a turbidity equivalent to a 0.5 McFarland standard (approximately 1.5 × 108 CFU/ml) using sterile phosphate-buffered saline

(PBS, pH 7.2). The optical density was verified spectrophotometrically at 625 nm (acceptable range: 0.08-0.13). This suspension was diluted 1:100 in MHB to obtain a final working inoculum of approximately 1.5 × 10^6 CFU/mL.

2.4. Antimicrobial Agents and Preparation

Vancomycin hydrochloride and Nortriptyline were purchased from Sigma-Aldrich Corp. (St Louis, MO, USA). Vancomycin stock solution was prepared in sterile distilled water, while Nortriptyline was dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in MHB. The final concentration of DMSO in any test well did not exceed 1% (v/v) .Solvent control wells containing MHB with 1% DMSO (without drugs) and growth control wells (bacteria without drugs) were included in all experiments to confirm that any observed inhibition was solely due to the antimicrobial agents. Stock solutions were prepared fresh on the day of testing, with aliquots stored at -70ºc for future use.

2.5. Determination of Minimum Inhibitory Concentration (MIC)

The MIC of each antimicrobial agent alone was determined using the broth microdilution method in 96-well microtiter plates according to CLSI guidelines(14). Briefly, two-fold serial dilutions of each agent were prepared in MHB. Each well received 50 µL of the bacterial inoculum, resulting in a final volume of 100 µL. Wells included: test wells (antimicrobial dilutions + inoculum), growth control (inoculum + MHB only), sterility control (MHB only), drug-only controls (drugs + MHB without bacteria). No color change was observed in these control wells, confirming that neither drug interfered with the resazurin indicator.

Plates were incubated at 37 ºC for 24 hours. Bacterial growth was assessed using the resazurin indicator (0.02% w/v); a color change from blue to pink indicated growth. The MIC was defined as the lowest concentration that completely inhibited visible growth (no color change). All tests were performed in triplicate and independently repeated on different days.

2.6. Checkerboard Assay for Synergy Testing

The synergistic interaction between vancomycin and Nortriptyline was evaluated using the checkerboard method in 96-well microtiter plates. Two-fold serial dilutions of vancomycin were prepared along the horizontal axis (abscissa). In contrast, two-fold serial dilutions of Nortriptyline were prepared along the vertical axis (ordinate), creating a two-dimensional matrix of drug combinations. Each well received 50 µL of the bacterial inoculum (approximately 1.5 × 10^6 CFU/mL), resulting in a final volume of 100 µL per well. The plates were covered and incubated at 37ºC for 24 hours.

After incubation, bacterial growth was assessed using the resazurin indicator (0.02% W/V) as described above. The MIC of each drug in combination was defined as the lowest concentration that prevented the color change from blue to pink. The Fractional Inhibitory Concentration (FIC) index was calculated as follows: FICI= (MIC of drug A in combination/ MIC of drug A alone) + (MIC of drug B in combination/ MIC of drug B alone).

The interaction was interpreted as: synergism (FICI ≤ 0.5); additive (0.5< FICI≤1); indifferent (1< FICI ≤4); or antagonism (FICI > 4). The checkerboard assay was performed in triplicate and independently repeated on three separate days.

2.7. Data Analysis

The experiments were performed on three separate days, with each experiment conducted in triplicate. Given consistent results across all replicates, descriptive analysis was used to present the data.

3. Results

3.1. Antibacterial Susceptibility of Single Agents

The MICs of vancomycin and Nortriptyline against Staphylococcus aureus were determined by the broth microdilution method. The MIC of vancomycin was 7.81 µg/mL. According to CLSI guidelines, this MIC value categorized the test strain as Vancomycin-Intermediate S. aureus (VISA), indicating reduced susceptibility(14). The MIC of Nortriptyline, when tested alone, was 1000µg/mL, demonstrating intrinsic antibacterial activity, albeit at a high concentration.

3.2. Synergistic Interaction Between Vancomycin and Nortriptyline

The interaction between vancomycin and Nortriptyline was evaluated using the checkerboard assay. The MIC values for both compounds, alone and in combination, were summarized in Table 1. A fourfold reduction in the MICs of both agents was observed when combined. The MIC of vancomycin decreased from 7.81 µg/mL to 1.95 µg/mL. Similarly, the MIC of Nortriptyline decreased from 1000 µg/mL to 250 µg/mL in the presence of vancomycin.

The interaction was quantified by calculating the fractional Inhibitory Concentration index (FICI):

FIC of vancomycin (FICVAN) = MIC in combination / MIC alone=1.95/7.81=0.25

FIC of nortriptyline (FICNOR)= MIC in combination /MIC alone=250/1000=0.25

FICI= FICVAN + FICNOR= 0.25 + 0.25 = 0.5

According to the predefined interpretation criteria, an FICI of ≤0.5 indicated synergistic activity. This interaction was consistently observed across all independent experimental repetitions.

Table 1: MIC values of vancomycin and Nortriptyline alone and in combination against the clinical VISA isolate

Drug	MIC Alone (µg/mL)	MIC in Combination (µg/mL)	Fold Reduction
Vancomycin	7.81	1.95	4-fold
Nortriptyline	1000	250	4-fold
FICI			**0.5 (Synergistic)**

FICI: fractional inhibitory concentration index

4. Discussion

The escalating global burden of antimicrobial resistance highlights the urgent need for the development of innovative therapeutic strategies. The declining pipeline of new antibiotic classes, coupled with the rapid evolution of bacterial resistance, has underscored the inadequacy of conventional drug discovery approaches(15, 16). In this context, drug repurposing and the identification of existing non-antibiotic drugs with antimicrobial or antibiotic-adjuvant properties have emerged as a promising strategy to expand available therapeutic options. The present study investigated the in vitro antibacterial activity of the tricyclic antidepressant (TCA), Nortriptyline, alone and in combination with vancomycin, against a VISA isolate of Staphylococcus aureus.

Our findings demonstrate that Nortriptyline exhibits antibacterial activity against the tested isolate and enhances the in vitro activity of vancomycin, as evidenced by a 4-fold reduction in the MICs of both agents when combined and an FICI of 0.5. According to the predefined criteria, this interaction was considered consistent with synergy. A recent study by Cabral et al. (2025) reported antibacterial activity of Nortriptyline against S. aureus, with an MIC value of 128 µg/mL. It demonstrated an additive interaction between Nortriptyline and vancomycin. These findings provide additional support for Nortriptyline's antibacterial potential and further justify investigation of Nortriptyline–vancomycin interactions. However, these findings should be interpreted as preliminary in vitro evidence rather than evidence of clinical applicability at this stage. This observation aligns with a growing body of evidence supporting the antibacterial and adjuvant potential of tricyclic antidepressants (TCAs). Previous studies have reported antibacterial activity of nortriptyline and related TCAs against Gram-positive bacteria, particularly S, aureus (17). Although most available studies have investigated other TCAs such as amitriptyline, these findings provide a rationale for exploring structurally related compounds, including Nortriptyline. For instance, amitriptyline has shown efficacy against a wide range of Gram-positive and Gram-negative bacteria (18) and has demonstrated synergistic effects when combined with colistin against resistant Klebsiella pneumoniae (19). Further supporting this concept, the combination of amitriptyline with tetracycline proved effective against multidrug-resistant Acinetobacter baumannii (20). At the same time, the TCA amoxapine resensitized MRSA to oxacillin, potentially by inhibiting beta-lactamase activity (21). Collectively, these studies support the potential role of antidepressant compounds as antibiotic adjuvants warranting further investigation.

While this study provides preliminary in vitro evidence for the vancomycin-nortriptyline interaction, several limitations should be considered. First, the findings are based on a single VISA isolate; therefore, they cannot be generalized to other VISA populations and require validation using a larger collection of genetically diverse clinical isolates. Furthermore, the high MIC of Nortriptyline alone (1000 µg/mL) represents a potential limitation for its further development as an antimicrobial agent. It requires additional evaluation of its pharmacological feasibility and safety. Although combination with vancomycin reduced the nortriptyline MIC in vitro, the clinical relevance of this finding remains uncertain. It requires further pharmacokinetic, pharmacodynamic, and toxicity evaluations.

Additionally, although the checkerboard assay is a widely used method for initial evaluation of antimicrobial interactions, time-kill studies and additional validation approaches were not performed in this study and should be considered in future investigations. Therefore, these findings should not be interpreted as evidence for direct clinical use at this stage. Given its reported antimicrobial properties, Nortriptyline may represent a candidate for further exploration; however, additional studies are necessary to evaluate its feasibility and safety.

Scientific literature suggests that TCAs may exert antimicrobial effects through multiple mechanisms. Proposed mechanisms include induction of oxidative stress, effects on bacterial genetic material, disruption of membrane integrity, and modulation of bacterial efflux systems. However, the relative contribution of these mechanisms appears to vary among different TCAs and bacterial species(12, 13). Nevertheless, the precise mechanisms

underlying the interaction between Nortriptyline and vancomycin observed in the present study remain unclear and require further experimental investigation.

From a translational perspective, future studies should include multiple VISA isolates, time-kill kinetics, mechanistic investigations, and in vivo models to determine whether the observed in vitro interaction can be reproduced under more clinically relevant conditions.

5. Conclusion

In conclusion, our results provide preliminary evidence that Nortriptyline can enhance the in vitro activity of vancomycin against a VISA isolate of S. aureus. These findings support further investigation of Nortriptyline as a potential antibiotic adjuvant, while emphasizing that substantial additional validation is required before considering clinical application.

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