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RESEARCH ARTICLE

Alharbi et al: 3-HQX-2thiol & vancomycin synergy

Unveiling the synergistic power of 3-hydrazinoquinoxaline-2-thiol and vancomycin against MRSA: An *in vitro* and *in silico* evaluation

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen causing infections ranging from skin disorders to severe conditions like infective endocarditis. Its evolving resistance, including resistance to β-lactams and last-resort antibiotics such as vancomycin, daptomycin, and linezolid, necessitates alternative therapies. This study investigates the synergistic efficacy of vancomycin and 3-hydrazinoquinoxaline-2-thiol (3HL) against 23 clinical MRSA isolates. Susceptibility testing was performed using broth microdilution and checkerboard assays, while in silico analyses assessed interactions between vancomycin and 3HL. Vancomycin exhibited minimum inhibitory concentrations (MICs) ranging from 0.25 to 1 µg/mL, whereas 3HL showed higher MICs of 16 to 32 µg/mL. Synergistic interactions were confirmed via checkerboard assays, with fractional inhibitory concentration index (FICI) values between 0.236 and 0.5, indicating enhanced vancomycin efficacy. Notably, vancomycin MICs decreased significantly when combined with 3HL. In silico docking revealed interactions with Penicillin-Binding Protein 2a (PBP2a), suggesting promising therapeutic potential. Vancomycin exhibited superior docking scores (-8.9 kcal/mol) and stabilizing hydrogen bonds, effectively targeting key protein grooves. Both compounds demonstrated potential for overcoming PBP2a's structural occlusions, suggesting their role in combating β-lactam-resistant strains through targeted protein inhibition and structural stabilization.

Keywords: MRSA; vancomycin; 3-Hydrazinoquinoxaline-2-thiol; 3HL; combination therapy; *in silico* analysis.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a versatile pathogen responsible for a range of infections, from superficial skin conditions to severe diseases such as infective endocarditis [1]. Its remarkable adaptability has made it a significant threat to antimicrobial resistance (AMR) [2]. Initially resistant to penicillin due to β-lactamase production, *S. aureus* later developed resistance to most β-lactams through the acquisition of the *mecA* gene, encoding penicillin-binding protein 2a (PBP2a) [3], [4]. Alarmingly, resistance has now extended to last-resort antibiotics, including vancomycin, daptomycin, and linezolid, complicating treatment options [5]. This growing resistance necessitates exploring alternative strategies, such as combination therapies and novel inhibitors, to combat *S. aureus*-associated infections effectively [6].

Vancomycin, while widely recognized as the gold standard therapy for infections caused by Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), has notable limitations despite its proven efficacy [7]. Although it is effective in many clinical scenarios, its bactericidal activity is relatively low compared to other antibiotics, which limits its ability to rapidly eliminate bacterial populations. Additionally, vancomycin exhibits a slow killing rate, which can prolong the duration of therapy and increase the risk of complications associated with persistent infections [8]. One of the most critical challenges is its inability to effectively target biofilms when used alone [9]. Biofilms, which are structured communities of bacteria encased in a protective extracellular matrix, are notoriously difficult to eradicate due to their reduced susceptibility to antimicrobial agents [10]. Furthermore, the emergence of vancomycin resistance has been frequently reported in clinical practice, posing a significant threat to its continued utility as a monotherapy [8]. These factors collectively highlight the need for combination therapies or alternative strategies to address infections, particularly those involving biofilms or resistant bacterial strains.

Combination therapy offers several significant advantages over monotherapy, even in cases where a single drug might demonstrate effective activity [11]. By employing two synergistic drugs, the required dose of each agent can often be reduced, which in turn minimizes the risk of dose-dependent toxicity and improves the overall safety profile of the treatment [12]. This is particularly beneficial in managing infections that require prolonged therapy or in treating vulnerable patient populations where high drug toxicity is a major concern [13]. Furthermore, combination therapy reduces the likelihood of the development of resistance, as pathogens face multiple simultaneous mechanisms of attack, making it more difficult for them to adapt and survive [14].

Additionally, synergistic combinations can enhance the bactericidal activity of the treatment, leading to faster and more effective elimination of pathogens [15]. This is particularly crucial in severe or complicated infections, where rapid bacterial clearance can significantly impact patient outcomes [16]. Moreover, combination therapy can improve the delivery and penetration of drugs into challenging infection sites, such as biofilms or tissues with poor vascularization, where monotherapy may fail to achieve adequate concentrations [17]. Collectively, these benefits make combination therapy a powerful strategy in combating infections, particularly in an era of increasing antimicrobial resistance and the growing complexity of infectious diseases.

Using in silico methods and molecular docking to identify possible drug targets is a crucial approach in modern drug discovery and development [18]. These computational techniques offer several significant advantages. In silico approaches can predict and analyze biological targets by mining databases, identifying conserved regions, and evaluating their potential as druggable sites [19]. This provides an efficient way to focus on high-value targets [20]. Docking studies simulate the interaction between a potential drug molecule and its target, revealing binding affinities, interaction sites, and key residues involved [21]. This helps in understanding the mechanism of action at the molecular level [22]. Conducting *in vitro or in vivo* experiments for screening potential drug candidates can be expensive and time-consuming [23]. In silico methods allow for the rapid screening of thousands of compounds, significantly reducing the resources required [24]. Docking results can guide the rational design of new compounds by providing insights into optimizing binding affinities and enhancing selectivity for the target, leading to more effective drugs with fewer side effects [25]. Docking studies can identify potential off-target effects, aiding in the design of molecules that are more specific to the target, which is especially valuable for personalized medicine approaches [26].

Recently, 3HL has emerged as a promising compound with notable antimicrobial properties. Its efficacy has been demonstrated not only against bacterial pathogens but also against fungal species such as *Candida*, highlighting its broad-spectrum potential [27], [28]. In addition to its inherent antimicrobial activity, studies have shown that this compound can synergize with penicillin to enhance its activity against methicillin-resistant *Staphylococcus aureus* (MRSA), showcasing its potential role in combination therapy [28].

However, despite its demonstrated benefits, no research to date has explored the efficacy of 3HL in combination with vancomycin, the standard therapy for MRSA infections. This

represents a significant gap in the current understanding of how this compound might complement vancomycin's bactericidal mechanisms to overcome resistance challenges. Therefore, this study aims to (1) evaluate the synergistic potential of 3HL and vancomycin against MRSA through in vitro antimicrobial assays, (2) investigate the mechanism of interaction using in silico molecular docking analysis, and (3) assess the impact of this combination on key MRSA resistance targets. These findings could pave the way for innovative therapeutic strategies against this formidable pathogen.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Clinical isolates of MRSA were obtained from the microbiology department of King Abdulaziz University Hospital (KAUH). All isolates were confirmed using standard microbiological methods and stored at −80°C in 15% glycerol until further use. For all experiments, the isolates were cultured either in blood agar or Mueller-Hinton agar and incubated at 37°C under aerobic conditions.

Determination of minimum inhibitory concentration (MIC)

The MICs of vancomycin and the 3HL were determined using the broth microdilution method, following Clinical and Laboratory Standards Institute (CLSI) guidelines [29], [30].

To determine the initial concentrations of vancomycin and the quinoxaline derivative and prepare their serial dilutions, the equation $C1V1=C2V2$ can be employed. This method ensures precise preparation of the desired concentrations for MIC and FIC studies.

The volume is taken from the stock solution and diluted with Mueller-Hinton Broth (MHB). Serial two-fold dilutions are performed to achieve a range of concentrations. Then, 100 µL of the solution from the previous well is transferred to the next well. This process is repeated across the plate, creating a gradient of concentrations for each compound. Serial two-fold dilutions of each compound were prepared in MHB and added to 96-well plates containing standardized bacterial suspensions at a final concentration of ~5×10^5 CFU/mL. Plates were incubated at 37°C for 24 hours, and the MIC was recorded as the lowest concentration of the drug that inhibited visible growth [5]. To ensure the accuracy and reliability of the MIC determinations, appropriate controls were included in all experiments. A negative control consisting of media alone was used to confirm sterility, while a positive control (bacteria in media without antibiotics) was included to verify bacterial viability. These controls ensured that the lowest concentration inhibiting bacterial growth was solely due to the antimicrobial

activity of the tested compounds. For synergy assessment, the MIC of each drug vancomycin and 3HL was determined individually and in combination using the checkerboard assay. Monotherapy for each drug was tested separately, and the results were compared to the combination therapy.

Checkerboard assay for combination studies

The interaction between vancomycin and the 3HL was evaluated using a checkerboard microdilution assay. Serial dilutions of vancomycin were prepared along the horizontal axis of a 96-well plate, while dilutions of the 3HL were prepared along the vertical axis. Each well contained a combination of both compounds in varying concentrations, along with a bacterial inoculum of ~5×10^5 CFU/mL. Plates were incubated at 37°C for 24 hours.

The Fractional Inhibitory Concentration Index (FICI) was calculated using the formula:

FICI=(MIC of drug A in combination÷MIC of drug A alone)+(MIC of drug B in combination ÷MIC of drug B alone) [14]

The interaction was interpreted as:

Synergy: FICI ≤ 0.5

Additive: 0.5 < FICI ≤ 1

Indifference: 1 < FICI ≤ 4

Antagonism: FICI > 4

In silico analysis

In this study, in silico methods is used to assess the possible synergetic effects of vancomycin and 3HL against PBP2a from MRSA. The crystal structure of PBP2a from MRSA in complex with piperacillin at the active site (PDB ID: 6H5O), was downloaded from the PDB database (https://www.rcsb.org/structure/6H5O). PubChem database was used to obtain the 3D structure of vancomycin (ID: 14969) and 3HL (ID: 781248). Crystal structures were prepared before docking by the addition of hydrogen bonds, removal of water molecules from protein, and energy minimization by the Mastro tool (2021). Site Map tool of Mastro interface was used for the prediction of active sites in PBP2a. Extra Precision docking of the Mastro tool was employed to study the possible interaction between the compounds and the protein active site, the MMGBSA analysis was employed to estimate the delta G (dG) gendered from binding of the complex. The generated complexes were analysed for types and length of bonds and associated between the interaction of compounds and protein, using PLIP (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index). The PyMOL molecular graphic system v 2.5.8, was used for visualization of 3D interactions.

Interpretation of results

All experiments were performed in triplicate, the average was calculated and results were expressed as the mean MIC and FICI values.

RESULTS

MICs of vancomycin and 3HL against MRSA strains

The MIC values for vancomycin against 23 MRSA strains ranged from 0.25 µg/mL to 1 µg/mL, with most strains showing MIC values of 0.5 µg/mL and 1 µg/mL. In contrast, the MIC values for the 3HL were uniformly higher, ranging from 16 µg/mL to 32 µg/mL across the tested strains. Notably, vancomycin demonstrated lower MIC values, indicating higher potency against the MRSA strains when compared to the 3HL. Strains such as MRSA 7 showed the lowest MIC for vancomycin (0.25 µg/mL), while MRSA 105 and MRSA 106 displayed varied MICs of 0.5 µg/mL and 1 µg/mL, depending on the strain. The consistency in the 3HL MICs across the majority of strains (32 µg/mL) suggests a limited variability in its activity. These findings highlight the differences in susceptibility patterns between vancomycin and the 3HL against MRSA. (Table 1).

The interaction between vancomycin and *3HL* was evaluated against 23 clinical MRSA isolates using checkerboard assays to determine their combined effects. The FICI values for the tested

isolates ranged from 0.236 to 0.5, with an average FICI across all isolates calculated at 0.332, indicating a thoroughly synergistic interaction. Notably for vancomycin, the MIC values decreased significantly when combined with *3HL*, indicating enhanced potency of vancomycin in the presence of 3HL. For example, MICs of vancomycin reduced from 1 μg/mL to as low as 0.06 μg/mL for several isolates. On the other hand, a majority of isolates exhibited FICI values below 0.5 (Table 2), confirming synergistic interactions, while a few showed values close to 0.5. No antagonism was observed. While slight variations in FICI were noted among different isolates, the overall trend indicated a synergistic interaction between vancomycin and 3HL, especially for isolates 101, 92, and 54, which consistently demonstrated FICI values in the synergistic range. This study highlights the potential of *3HL* derivatives to enhance the antimicrobial activity of vancomycin against MRSA and underscores the importance of further investigations to optimize the combination for clinical application.

Vancomycin and 3HL predicted to efficiently inhibit the active and allosteric sites of PBP2a

In this study, an in silico approach was used to predict possible inhibitors for PBP2a from MRSA, a protein essential for cell wall biosynthesis [31]. Screening of protein pockets revealed the presence of five pockets with values mostly above 1.0 Å except for site 5 (0.756) (Table 3, Figure 1), suggesting favourable binding, in general pockets with Dscore values >0.98 considered as druggable [32]. The known active site of PBP2a is shown in figure 1, and indicated by "B", while the allosteric site is indicated by "A" [33], [34]. It has been proposed that the active site of PBP2a cannot be inhibited by β-lactams due presence of protective loops souring this site [34], blocking of the allosteric site proved to be effective in treatment of resistant bacteria, which is associated by opening of the active site resulting in blocking the active site [34].

In this study, vancomycin and 3HL effectively blocked both the active and allosteric sites, as shown in Table 3 and Figure 2. Vancomycin (ID: 14969) exhibited the best docking score of -8.9 kcal/mol and a dG bind of -56 kcal/mol when interacting with the allosteric site (A). Additionally, it efficiently blocked two other grooves (C and D) with docking scores of -7.8 and -10.8, respectively. This finding suggests that vancomycin may promote the opening of the protein's active site and enhance its stability, preventing twisting and closure. Meanwhile, 3HL interacted with the protein's active site, yielding a docking score of -4.9 kcal/mol and a dG bind energy of -37 kcal/mol (Table 3).

Table 4 and figure 3, summarize the interacting residues of compounds, and various grooves of the PBP2a. Vancomycin interacted with multiple residues across different grooves, including ASN, TYR, THR, GLU, and others. It demonstrates strong interactions with donor-acceptor distances ranging between 1.67 Å and 3.45 Å. While, the 3HL (ID: 781248) interacted with residues like SER, GLN, HIS, LYS, and ASN in various grooves. Vancomycin interacts with residues such as ASN606 and THR373 (within or near the 594–603 region) [34], which could help overcome the distortion by forming stable hydrogen bonds (e.g., 1.87 Å distance with ASN606). This interaction might contribute to vancomycin's potential to open the active site by stabilizing its structure and preventing loop-mediated occlusion.

DISCUSSION

The results of this study revealed that vancomycin demonstrated potent activity against *Staphylococcus aureus* clinical MRSA isolates, with MIC values ranging from 0.25–1 µg/mL. However, the 3HL exhibited relatively higher MICs (16–32 µg/mL). Checkerboard assays showed a synergistic interaction between the two compounds, with FICI values ranging from 0.236 to 0.5. Notably, vancomycin MICs significantly decreased in combination with the 3HL. This study highlights the synergistic efficacy of vancomycin and 3HL against MRSA, addressing a critical gap in antimicrobial resistance research. While all strains demonstrated synergistic effects, the FICI values ranged from 0.23 to 0.45, indicating variability in the degree of synergy. This suggests that some strains responded more favorably to the combination treatment than others. These findings highlight a novel strategy to enhance the potency of existing antibiotics while exploring complementary mechanisms of action.

The combination of vancomycin and the 3HL offers multiple therapeutic advantages. Vancomycin's MICs were substantially reduced in the presence of the 3HL, demonstrating enhanced efficacy against MRSA strains. This reduction not only signifies synergy but also underscores the potential for reduced dosing, potentially minimizing adverse effects [12]. The combination may also offer an effective means to overcome biofilm-related challenges and persistent bacterial infections, which are notoriously difficult to treat with monotherapy [35].

2,3-Dimethylquinoxaline (DMQ) is recognized as a broad-spectrum antimicrobial phytochemical. This study evaluates its toxicological profile through both in vitro and in vivo methods. Cardiotoxicity, nephrotoxicity, and hepatotoxicity were assessed in cell cultures, while acute oral toxicity (AOT) and subacute oral toxicity (SAOT) were evaluated in mice. Acute dermal toxicity (ADT) tests were conducted in rats. In vitro tests showed no significant

toxicity at concentrations up to 100 μM, except for a slight, non-significant ATP reduction in human hepatocellular carcinoma cells. The median lethal dose (LD50) of DMQ was above 2000 mg/kg, with no mortality or clinical abnormalities observed in animals. Biochemical analysis indicated increased platelet and white blood cell counts by 99.8% and 188.8%, respectively, in treated groups. Histological findings included enlarged renal corpuscles, hyperplasia of testosterone-secreting cells, and coronary and capillary dilation. Overall, DMQ demonstrated an acceptable safety profile in rodents, although high doses caused thrombocytosis, leukocytosis, and tissue alterations, warranting further investigation [36]. Given the structural similarity between 3HL and DMQ, it is reasonable to hypothesize that 3HL may exhibit a comparable safety profile.

In silico docking studies provided further insight into the molecular basis of the observed synergy. Both vancomycin and the 3HL effectively targeted key binding sites in PBP2a, a critical enzyme in MRSA's resistance mechanism. Vancomycin exhibited superior binding affinity (-8.9 kcal/mol) through stabilizing hydrogen bonds, while the 3HL also demonstrated significant interactions. These findings suggest that the combination targets complementary sites within the protein, potentially amplifying the antimicrobial effects through structural inhibition and stabilization.

Our in silico docking studies revealed that vancomycin forms stabilizing hydrogen bonds with residues *ASN, TYR, THR, GLU,* and others, demonstrating strong interactions with donor-acceptor distances ranging from 1.67 Å to 3.45 Å. Similarly, 3HL (ID: 781248) interacts with residues such as *SER, GLN, HIS, LYS,* and *ASN* in various grooves of PBP2a, reinforcing the inhibition of its activity. Vancomycin interacts with critical residues, including *ASN606* and *THR373* (within or near the *594–603* region), which have been reported to play a role in the enzyme's function. These interactions, particularly the hydrogen bond formation (e.g., 1.87 Å distance with ASN606), may contribute to overcoming structural distortions by stabilizing the active site and preventing loop-mediated occlusion. The complementary binding patterns of vancomycin and 3HL suggest that the combination therapy may exert its synergistic effect by targeting distinct yet functionally relevant regions of PBP2a, ultimately disrupting its catalytic function and enhancing antimicrobial efficacy.

While the docking scores and MMGBSA values indicate favorable binding affinities of 3HL and vancomycin with the *mecA* protein, it is essential to recognize the limitations of these computational predictions. In silico methods, though valuable for providing preliminary

insights into potential molecular interactions, do not fully account for the dynamic and complex environment within living organisms, such as protein flexibility, cellular uptake, metabolism, and the influence of other biomolecules [37]. Furthermore, high binding affinity in computational models does not always translate to corresponding biological activity *in vitro* or *in vivo* [38], [39]. Therefore, while our docking results support the potential synergistic effect of the drug combination, these findings must be validated through further experimental studies to confirm their biological significance and therapeutic potential.

The combination exploits distinct mechanisms of action: vancomycin inhibits bacterial cell wall synthesis by targeting D-Ala-D-Ala termini, disrupting peptidoglycan crosslinking [40], while 3HL inhibits DNA synthesis and promotes reactive oxygen species (ROS) production [41], [42]. This dual mechanism may explain the enhanced bactericidal activity, as it addresses different aspects of bacterial survival and resistance. The ROS production by 3HL adds an oxidative stress component, further weakening the pathogen's defenses.

The combination of vancomycin and 3-Hydrazinoquinoxaline-2-thiol (3HL) presents a promising therapeutic strategy in addressing the growing challenge of methicillin-resistant *Staphylococcus aureus* (MRSA) infections. Vancomycin has long been a cornerstone in the treatment of MRSA; however, the emergence of vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and VRSA) strains has significantly limited its clinical efficacy [43]. Our findings suggest that adding 3HL enhances vancomycin's antibacterial activity, potentially through synergistic mechanisms that disrupt bacterial cell wall synthesis or target alternative pathways, thereby overcoming vancomycin resistance. This highlights the need to investigate the activity of this combination against VISA and VRSA strains.

Importantly, MRSA-associated infections are frequently complicated by biofilm formation, which further exacerbates antibiotic resistance and complicates treatment. Biofilms act as a protective barrier, reducing antibiotic penetration and shielding bacteria from the host immune response [44]. The ability of the vancomycin-3HL combination to combat biofilm-associated MRSA infections could represent a critical advancement in clinical treatment, highlighting the need to assess its effectiveness in improving bacterial clearance in biofilm-forming MRSA strains.

Further *in vivo* studies are warranted to confirm the efficacy of this combination therapy in biofilm-associated MRSA infections. Understanding the pharmacokinetics and pharmacodynamics of this combination will also be essential to optimize dosing regimens and

maximize therapeutic outcomes. If successful, this novel approach could offer a valuable alternative for clinicians facing multidrug-resistant MRSA infections, particularly in cases where conventional therapies have failed.

By addressing both vancomycin resistance and biofilm-associated challenges, our study contributes to the development of innovative therapeutic strategies aimed at mitigating the growing threat of antibiotic-resistant pathogens in clinical settings. Moreover, future work should focus on evaluating the combination in vivo to confirm efficacy and safety profiles, particularly in animal infection models. Time-kill assays studies will be critical to understanding the kinetics of bacterial eradication and efficacy in biofilm-associated infections. Additionally, toxicity studies are essential to ensure the safety of 3HL and their compatibility with vancomycin for clinical application.

CONCLUSION

This study demonstrates the synergistic efficacy of vancomycin and 3HL against MRSA, providing a novel combination therapy that enhances antimicrobial activity while potentially reducing resistance. The distinct and complementary mechanisms of action offer a promising strategy for addressing β-lactam-resistant bacteria. Future investigations into in vivo efficacy, biofilm activity, and toxicity are warranted to translate these findings into clinical practice.

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REFERENCES

1- Fowler, V. G., Miro, J. M., Hoen, B., Cabell, C. H., Abrutyn, E., Rubinstein, E., ... & ICE Investigators, F. T. Staphylococcus aureus endocarditis. JAMA 2005;293(24):3012–21.
2- Somerville GA. Staphylococcus: genetics and physiology. 2016:390.
3- Ballhausen B, Kriegeskorte A, Schleimer N, Peters G, Becker K. The mecA homolog mecC confers resistance against β-lactams in Staphylococcus aureus

irrespective of the genetic strain background. Antimicrob Agents Chemother 2014;58(7):3791–8.
4- García-Álvarez L, Holden MTG, Lindsay H, et al. Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect Dis 2011;11(8):595–603.
5- Elfadil A, Ibrahem K, Abdullah H, Mokhtar JA, Al-Rabia MW, Mohammed HA. Synergistic activity of 3-hydrazinoquinoxaline-2-thiol in combination with penicillin against MRSA. Infect Drug Resist 2024;17:355–64.
6- Ba X, Harrison EM, Edwards GF, et al. Old drugs to treat resistant bugs: Methicillin-resistant Staphylococcus aureus isolates with mecC are susceptible to a combination of penicillin and clavulanic acid. Antimicrob Agents Chemother 2015;59(12):7396–404.
7- Tarai B, Das P, Kumar D. Recurrent challenges for clinicians: Emergence of methicillin-resistant Staphylococcus aureus, vancomycin resistance, and current treatment options. J Lab Physicians 2013;5(2):71–8.
8- Zimmerli W, Frei R, Widmer AF. Microbiological tests to predict treatment outcome in experimental device-related infections due to Staphylococcus aureus. J Antimicrob Chemother 1994;33:959–67.
9- Warren PT, Rose E. Impact of biofilm on the in vitro activity of vancomycin alone and in combination with tigecycline and rifampicin against Staphylococcus aureus. J Antimicrob Chemother 2008;16(1):1–23.
10- Murugaiyan J, Ranjan K, Arul V, et al. Progress in alternative strategies to combat antimicrobial resistance: Focus on antibiotics. Antibiotics 2022;11(2):1–37.
11- Tamma PD, Cosgrove SE, Maragakis LL. Combination therapy for treatment of infections with Gram-negative bacteria. Clin Microbiol Rev 2012;25(3):450–70.
12- Hu A, Liu Y, Coates AR. Azidothymidine produces synergistic activity in combination with colistin against antibiotic-resistant Enterobacteriaceae. Antimicrob Agents Chemother 2019;63(1):1–11.
13- Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ. Antimicrobial host defence peptides: functions and clinical potential. Nat Rev Drug Discov 2020;19(5):311–32.
14- Gonzales, P. R., Pesesky, M. W., Bouley, R., Ballard, A., Biddy, B. A., Suckow, M. A., ... & Dantas, G. Synergistic, collaterally sensitive β-lactam combinations suppress resistance in MRSA. Nat Chem Biol 2015;11(11):855–61.
15- Hackbarth CJ, Chambers HF, Sande MA. Serum bactericidal activity of rifampin in combination with other antimicrobial agents against Staphylococcus aureus. Antimicrob Agents Chemother 1986;29(4):611–3.
16- Kalghatgi S, Spina CS, Costello JC, et al. Bactericidal antibiotics induce mitochondrial dysfunction and oxidative damage in mammalian cells. Sci Transl Med 2013;5(192).
17- Davis JS, Van Hal S, Tong SYC. Combination antibiotic treatment of serious methicillin-resistant Staphylococcus aureus infections. Semin Respir Crit Care Med 2015;36(1):3–16.
18- Agamah FE, Ojo O, Olayemi I, et al. Computational/in silico methods in drug target and lead prediction. Brief Bioinform 2020;21(5):1663–75.

19- Koutsoukas A, Bender A, Reker D, et al. From in silico target prediction to multi-target drug design: Current databases, methods and applications. J Proteomics 2011;74(12):2554–74.
20- Nguyen M, Brettin T, Long SW, et al. Developing an in silico minimum inhibitory concentration panel test for Klebsiella pneumoniae. Sci Rep 2018;8(1):421.
21- Alonso H, Bliznyuk AA, Gready JE. Combining docking and molecular dynamic simulations in drug design. Med Res Rev 2006;26(5):531–68.
22- Okimoto N, Kinoshita T, Yamagata H, et al. High-performance drug discovery: Computational screening by combining docking and molecular dynamics simulations. PLoS Comput Biol 2009;5(10).
23- Wilkinson GF, Pritchard K. In vitro screening for drug repositioning. J Biomol Screen 2015;20(2):167–79.
24- Rao VS, Srinivas K. Modern drug discovery process: An in silico approach. J Bioinforma Seq Anal 2011;2(June):89–94.
25- Huggins DJ, Sherman W, Tidor B. Rational approaches to improving selectivity in drug design. J Med Chem 2012;55(4):1424–44.
26- Fan S, et al. Clarifying off-target effects for torcetrapib using network pharmacology and reverse docking approach. 2012;38.
27- Alkuwaity KIAE, Mokhtar JA, Abujamel T, Attallah D, Altorki TA, Alsieni M, Bazuhair MA, Helmi NR, AbdulMajed H, Juma NA, Abdullah H, Al-Rabia MW. Evaluation of the antibacterial activity of 3-hydrazinoquinoxaline-2-thiol compound against extended-spectrum beta-lactamases producing bacteria. Eur Rev Med Pharmacol Sci. 2024;28(10).
28- Bazuhair MA, Alkuwaity KIAE, Mokhtar JA, Attallah D, Altorki TA, Alsieni M, Helmi NR, AbdulMajed H, Juma NA, Abdullah H, Al-Rabia MW. The combination of 3-hydrazinoquinoxaline-2-thiol with thymoquinone demonstrates synergistic activity against different Candida strains. Infect Drug Resist. 2024;17:2289–2298.
29- Clinical and Laboratory Standards Institute (CLSI). M07-A9: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—Ninth edition. Available from: https://clsi.org/media/1928/m07ed11_sample.pdf. Accessed 2002.
30- Shalaby MAW, Dokla EM, Serya RAT, Abouzid KAM. Penicillin binding protein 2a: An overview and a medicinal chemistry perspective. Eur J Med Chem. 2020;199:2.
31- Mahmudpour M, Nabipour I, Keshavarz M, Farrokhnia M. Virtual screening on marine natural products for discovering TMPRSS2 inhibitors. Front Chem. 2021;9(October):1–14.
32- Otero, L. H., Rojas-Altuve, A., Llarrull, L. I., Carrasco-López, C., Kumarasiri, M., Lastochkin, E., ... & Hermoso, J. A. How allosteric control of Staphylococcus aureus penicillin binding protein 2a enables methicillin resistance and physiological function. Proc Natl Acad Sci U S A. 2013;110(42):16808–16813.
33- Chiang YC, Wong MTY, Essex JW. Molecular dynamics simulations of antibiotic ceftaroline at the allosteric site of penicillin-binding protein 2a (PBP2a). Isr J Chem. 2020;60(7):754–763.

34- Davis J, Hal S, Tong S. Combination antibiotic treatment of serious methicillin-resistant Staphylococcus aureus infections. Semin Respir Crit Care Med. 2015;36(1):3–16.
35- Alfadil, A., Alsamhan, H., Ali, A., Alkreathy, H., Alrabia, M. W., Fatani, A., & A. Ibrahem, K.. In-vivo and in-vitro toxicity evaluation of 2,3-dimethylquinoxaline: An antimicrobial found in a traditional herbal medicine. PLoS One. 2024;19(8):1–14.
36- Zhang X, Wu F, Yang N, Zhan X, Liao J, Mai S, Huang Z. In silico Methods for Identification of Potential Therapeutic Targets. Interdiscip. Sci. – Comput. Life Sci. 2022;14(2):285–310.
37- Di Ventura B, Lemerle C, Michalodimitrakis K, Serrano L. From in vivo to in silico biology and back. Nature. 2006;443(7111):527–533.
38- Taldaev A, Terekhov R, Nikitin I, Zhevlakova A, Selivanova I. Insights into the pharmacological effects of flavonoids: The systematic review of computer modeling. Int J Mol Sci. 2022;23(11).
39- Kwun MJ, Novotna G, Hesketh AR, Hill L, Hong HJ. In vivo studies suggest that induction of VanS-dependent vancomycin resistance requires binding of the drug to D-Ala-D-Ala termini in the peptidoglycan cell wall. Antimicrob Agents Chemother. 2013;57(9):4470–80.
40- Suter W, Rosselet A, Knuesel F. Mode of action of quindoxin and substituted quinoxaline-di-N-oxides on Escherichia coli. Antimicrob Agents Chemother. 1978;13(5):770–83.
41- Cheng G, Sa W, Cao C, Guo L, Hao H, Liu Z, et al. Quinoxaline 1,4-di-N-oxides: Biological activities and mechanisms of actions. Front Pharmacol. 2016;7(Mar):1–21.
42- Tartor YH, Enany ME, Ismail NI, El-Demerdash AS, Eidaroos NH, Algendy RM, et al. Vancomycin-resistant Staphylococcus aureus endangers Egyptian dairy herds. Sci Rep. 2024;14(1):1–14.
43- Ali A, Zahra A, Kamthan M, Husain FM, Albalawi T, Zubair M, et al. Microbial biofilms: Applications, clinical consequences, and alternative therapies. Microorganisms. 2023;11(8):1–28.

TABLES AND FIGURES WITH LEGENDS

Table 1. Interaction between vancomycin and 3HL against MRSA. The strains are listed by their identification numbers along with the corresponding MICs in µg/mL. The data provides an overview of the antimicrobial susceptibility of MRSA strains to the tested agents.

No	MRSA number	MIC Van µg/mL	MIC 3HL µg/mL
1	105	1	16
2	104	0.5	16
3	95	1	16
4	92	1	32
5	75	1	32
6	106	0. 5	16
7	101	1	32
8	98	0.5	32
9	97	1	32
10	100	0.5	32
11	109	1	32
12	7	0.25	32
13	80	1	16
14	92	1	32
15	73	1	32
16	54	1	32
17	34	0.5	32
18	1	0.5	32
19	2	0.5	32
20	3	0.5	32
21	4	0.5	32
22	11	0.5	32
23	9	0.5	32

Table 2. Fractional inhibitory concentration index (FICI) values and corresponding interaction interpretations for vancomycin and *3HL* against various MRSA strains. FICI values ≤ 0.5 indicate synergy, while values between 0.5 and 1.0 suggest an additive effect. This table highlights the predominant synergistic interactions between the two agents across the tested strains.

No	MRSA strain number	FICI	Interaction
1	105	0.437	Synergy
2	104	0.450	Synergy
3	95	0.360	Synergy
4	92	0.342	Synergy
5	75	0.373	Synergy
6	106	0.310	Synergy
7	101	0.350	Synergy
8	98	0.375	Synergy
9	97	0.350	Synergy
10	100	0.332	Synergy
11	109	0.332	Synergy
12	7	0.346	Synergy
13	80	0.360	Synergy
14	92	0.375	Synergy
15	73	0.236	Synergy
16	54	0.395	Synergy
17	34	0.290	Synergy
18	1	0.370	Synergy
19	2	0.413	Synergy
20	3	0.335	Synergy
21	4	0.352	Synergy
22	11	0.342	Synergy
23	9	0.332	Synergy

Table 3. Docking scores and MM GBSA dG bind of vancomycin (ID: 14969) and 3HL (ID: 781248) with different grooves in PBP2a

Site	Dscore	Volume	ID	XP docking	MM GBSA dG bind
A	1.018	527	14969	-8.9	-56
			781248	-3.9	-27
B	0.991	417	14969	-	-
			781248	-4.8	-37
C	1.011	375	14969	-7.8	-40
			781248	-3.3	-22
D	1.005	251	14969	-10.8	-50
			781248	-3.9	-31.9
D	0.756	151	14969	-	-
			781248	-3.1	-16.4

Table 4. Interacting residues of vancomycin (ID: 14969) and 3HL (ID: 781248) and with different grooves in PBP2a

Compound	Index	Residue	AA	Distance H-A	Distance D-A	Donor Angle	Donor Atom	Acceptor Atom
1_14969	1	120A	ASN	1.96	2.89	161.07	10311 [O3]	1981 [O2]
	2	170A	TYR	2.42	3.19	136.57	2777 [O3]	10326 [O2]
	3	190A	THR	2.43	3.02	119.29	3113 [O3]	10328 [O2]
	4	212A	THR	1.91	2.88	157.56	10340 [Nam]	3481 [O3]
	5	213A	GLU	1.76	2.78	177.59	10339 [N3]	3497 [O3]
	6	247A	LYS	2.28	3.03	129.45	4040 [N3+]	10312 [O3]
	7	249A	ASP	1.79	2.74	164.88	10312 [O3]	4073 [O-]
	8	346A	MET	1.78	2.72	161.79	10331 [O2]	5605 [O2]
1_781248	1	123A	SER	3.47	3.86	107.12	2035 [O3]	10310 [N3]
	2	266A	GLN	2.83	3.33	111.13	10307 [Nam]	4359 [O2]
	3	267A	HIS	3	3.93	151.65	10310 [N3]	4372 [O2]
	4	269A	ASP	2.71	3.71	171.49	4401 [Nam]	10309 [Npl]
2_781248	1	420A	TYR	2.17	3.18	173.27	10310 [N3]	6797 [O3]
	2	557A	HIS	3.08	3.91	134.71	8955 [Nar]	10308 [N2]
	3	616A	ALA	3.26	4.05	135.42	9880 [Nam]	10308 [N2]
	4	617A	SER	3.02	3.76	135.56	9895 [O3]	10310 [N3]
3_14969	1	163A	GLU	2.14	3.09	167.89	10314 [O3]	2667 [O2]

	2	165A	SER	2.34	2.82	110.22	2703 [O3]	10312 [O3]
	3	189A	LYS	2.22	3.14	150.99	3094 [N3+]	10314 [O3]
	4	193A	LYS	1.81	2.81	171.81	3168 [N3+]	10321 [O3]
	5	195A	ASP	3.25	4.08	140.99	10338 [Nam]	3206 [O.co2]
	6	196A	GLU	1.95	2.65	129.52	3218 [O3]	10328 [O2]
	7	196A	GLU	1.67	2.65	176.5	10328 [O2]	3218 [O3]
	8	197A	TYR	2.13	3.12	161.85	10339 [N3]	3237 [O3]
	9	350A	SER	3.06	3.55	110.62	5664 [Nam]	10312 [O3]
	10	350A	SER	2.77	3.52	135.82	5669 [O3]	10309 [O3]
	11	352A	GLU	1.88	2.87	160.97	10332 [N3]	5696 [O.co2]
	12	353A	GLU	2.23	3.15	159.83	10312 [O3]	5711 [O.co2]
3_781248	1	192A	LYS	3.08	3.95	145.74	3138 [Nam]	10308 [N2]
	2	193A	LYS	2.17	3.14	160.61	10309 [Npl]	3163 [O2]
	3	193A	LYS	2.47	3.12	121.92	3168 [N3+]	10310 [N3]
	4	195A	ASP	1.91	2.76	138.61	10310 [N3]	3206 [O.co2]
4_14969	1	225A	HIS	2.07	3	161.49	10314 [O3]	3675 [O2]
	2	237A	GLU	1.84	2.78	153.25	10339 [N3]	3859 [O.co2]
	3	240A	GLN	2.38	3.36	164.29	3915 [Nam]	10325 [O2]

	4	256A	GLY	3.45	4.04	122.21	10311 [O3]	4177 [O2]
	5	256A	GLY	2.43	3.17	129.13	4174 [Nam]	10308 [O3]
	6	340A	TYR	1.96	2.82	146.82	5509 [O3]	10315 [O3]
	7	358A	THR	2.07	2.95	150.86	10331 [O2]	5800 [O3]
	8	365A	LEU	2.15	2.86	129.54	10315 [O3]	5912 [O2]
	9	367A	ASN	2.37	3.26	147.12	5954 [Nam]	10312 [O3]
	10	370A	GLN	2.78	3.2	107.42	10312 [O3]	6010 [O2]
	11	370A	GLN	3.24	4.05	138.44	6011 [Nam]	10309 [O3]
4_781248	1	340A	TYR	2.16	2.88	130.94	11018 [O3]	20614 [N3]
	2	365A	LEU	1.91	2.81	144.73	20614 [N3]	11823 [O2]
	3	367A	ASN	3.11	3.99	146.24	11904 [N3]	20611 [Nam]
	4	367A	ASN	3.64	3.99	102.3	11908 [N3]	20611 [Nam]
	5	367A	ASN	3.46	4.09	122.37	11893 [N3]	20612 [N2]
	6	370A	GLN	1.86	2.71	139.34	20611 [Nam]	12016 [O2]
5_14969	1	373A	THR	2.92	3.85	162.41	6058 [O3]	10309 [Npl]
	2	606A	ASN	1.87	2.81	150.44	10309 [Npl]	9731 [O2]
	3	606A	ASN	2.75	3.51	131.98	9732 [Nam]	10310 [N3]

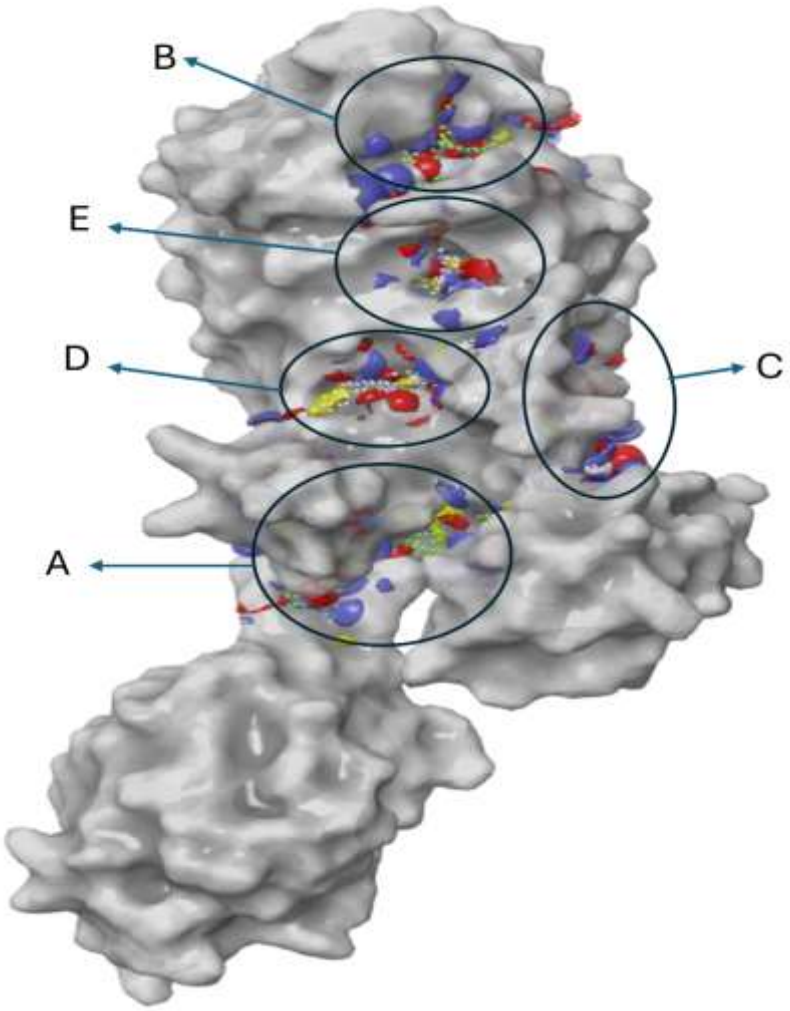

Figure 1. Modeled 3D structure of PBP2a from MRSA showing the pockets, the active site is indicated by "A", while the allosteric site is indicated by "B". While C, D, and E are other binding sites identified by SiteMap. The molecular interaction fields (yellow surface indicates hydrophobic, blue surface indicates hydrogen bond donor, red indicates hydrogen bond acceptor), and site-points (white spheres).

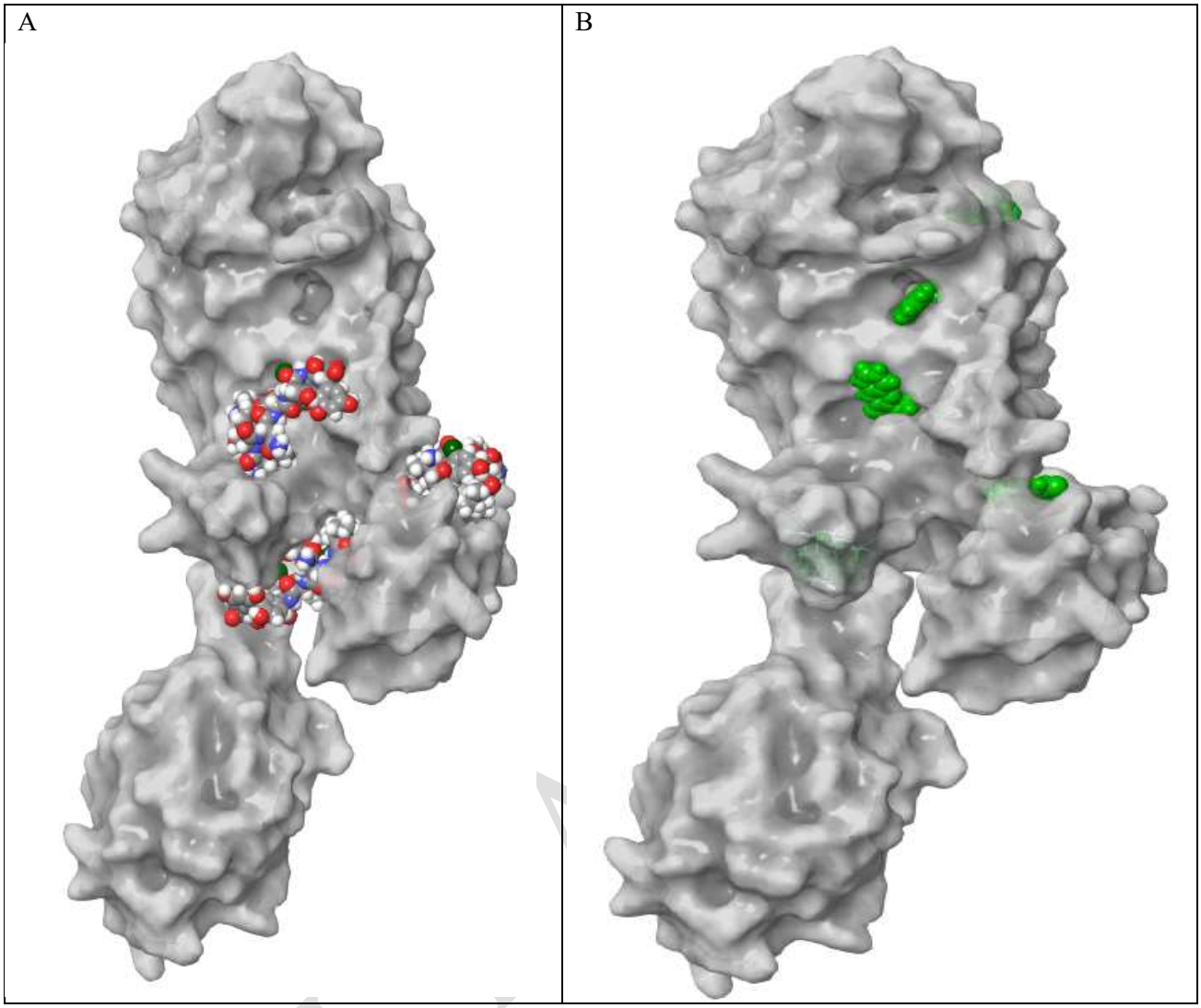

Figure 2. Interaction of PBP2a from MRSA and vancomycin (A) and 3HL (B). Vancomycin interacted with sites A, C, and D as shown in Figure 1. The 3HL interacted with all five sites. The precise amino acids involved in each site are presented in Table 4 and Figure 3.

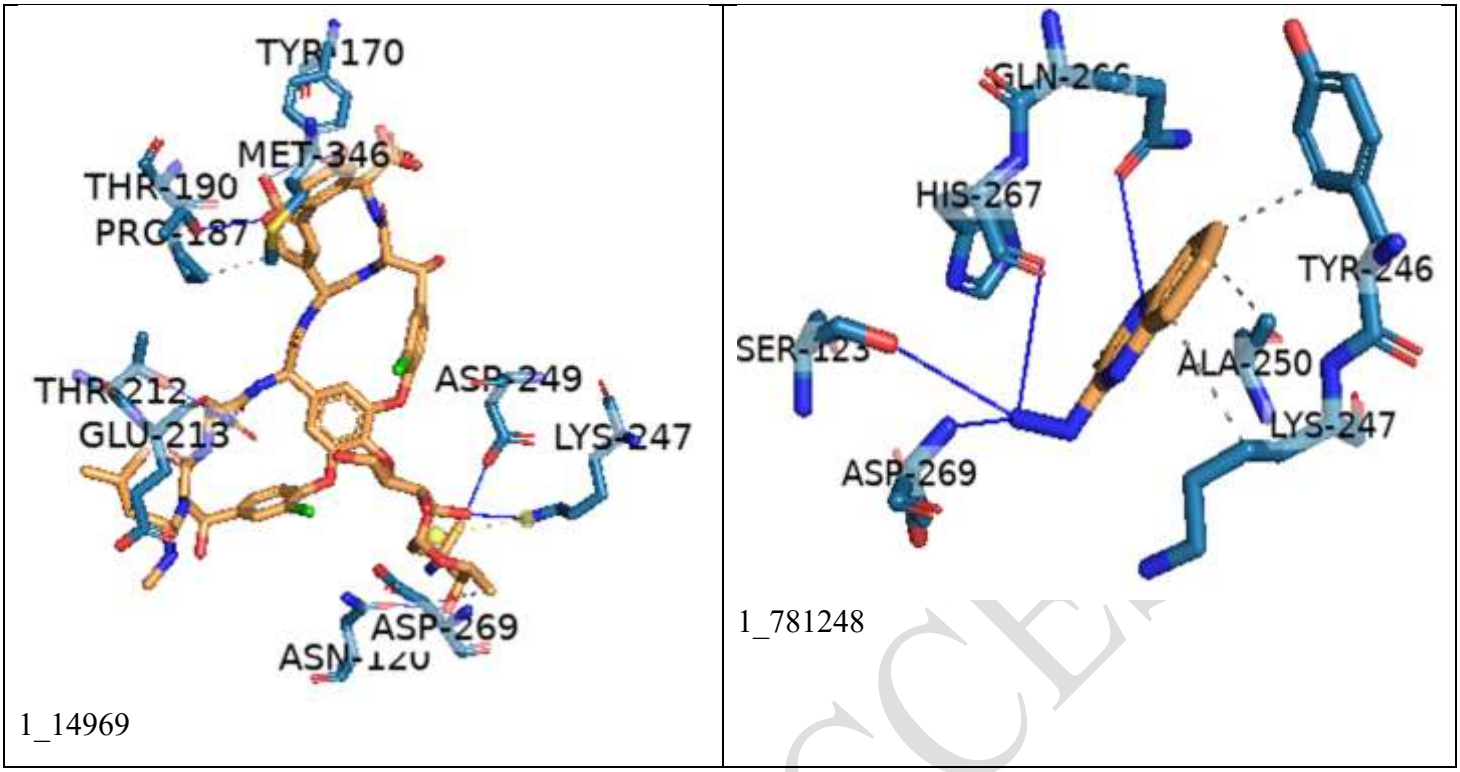

Figure 3. 3D interaction of vancomycin (ID: 14969) and *3*HL (ID: 781248) and with different grooves in PBP2a, the grooves are indicated by numbers as shown in Figure 1.