

## Elucidating the Molecular Determinants of High-Affinity Binding and Inhibition of *Staphylococcus aureus* NorA Efflux Pump: Its Implication for Drug Design

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### Abstract

Antimicrobial resistance (AMR) poses an important global health risk by diminishing the efficacy of existing antibiotics and complicating the treatment of bacterial infections. A key resistance mechanism involves bacterial efflux pumps, such as NorA, which expel diverse antimicrobial agents including fluoroquinolones, lowering intracellular drug concentrations and reducing activity. Efflux pump inhibitors (EPIs) can restore antibiotic potency by blocking these pumps. Although reserpine is a known NorA inhibitor, its clinical use is limited by adverse effects such as depression and neurotoxicity. Understanding molecular interactions that confer high-binding affinity to NorA is essential for designing potent and less toxic EPIs. The 3D crystal structure of NorA efflux pump was retrieved from Protein Data Bank (PDB ID: 7LO8). The protein was prepared for docking using BIOVIA Discovery Studio and AutoDockTools (ADT) following standard protein preparation protocol for molecular docking with AutoDockVina. In a similar way, ligands were retrieved from PubChem in SDF (Structure Data File) format and prepared for docking. They were energy-minimized on Avogadro, and Gasteiger charges were computed and torsions were defined on ADT. Data was analyzed based on binding affinity and interactions with relevant residues in the binding pocket of NorA. Reserpine demonstrated a significantly stronger binding affinity of -9.5 kcal/mol compared to -8.9kcal/mol for oleandomycin. Ciprofloxacin exhibited the lowest binding affinity of -8.3 kcal/mol. Reserpine formed extensive interactions with the pocket residues and with key residues (Glu 222, Asp 307 and Arg 310). The study revealed key interactions that confer high-affinity binding to the pocket of NorA efflux pump, offering a basis for designing more potent and safer EPIs.

**Keywords:** antibiotic, resistance, efflux pump, reserpine, molecular docking, drug design

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### Introduction

Multidrug resistance (MDR) arises when microorganisms, especially bacteria, develop different mechanisms of evading antibiotics, thereby gaining the ability to resist multiple drugs simultaneously. These mechanisms include efflux pumps, drug-modifying enzymes, altered targets, or reduced uptake. This is clinically significant because it reduces the efficacy of therapeutic interventions. The global increase in MDR bacteria, such as methicillin-

resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinobacterbaumannii*, has become a major clinical and public health challenge, which emphasizes the need for effective and novel antimicrobial drugs (Parmanik et al., 2022; Abebe & Birhanu, 2023; Kunz Coyne et al., 2022; Ibrahim et al., 2021).

Efflux pumps are protein transporters that actively expel substances out of the cell. Microorganisms utilize these mechanisms to actively remove toxic compounds, including

antibiotics, from the cell interior. This expulsion results in a decreased intracellular concentration of the antimicrobial agent. In *Staphylococcus aureus*, there are at least ten different types of pumps, and the major and most efficient of them all is the NorA efflux pump (Monteiro et al., 2020). There are at least five major superfamilies of efflux pump proteins and NorA is classified within the Major Facilitator Superfamily (MFS) (Li et al., 2024). It is an integral protein comprising of 388 amino acids and 12 transmembrane helices (Shang et al., 2021; Costa et al. 2019; de Moraes et al., 2023). The NorA efflux pump is a proton motive force. This force is generated by the bacterial electron chain to create a proton gradient across the cell. To expel a drug or substrate, the drug must bind to the internal binding site of NorA, while proton from the periplasm or extracellular environment binds to NorA. The binding of proton triggers a conformational change of the 12-helix transmembrane protein; subsequently, the internal binding site containing the substrate is exposed to the outside environment and is expelled. Important residues involved in substrate recognition and binding include Ile23, Phe140, Glu222, Tyr225, Phe303, Ile 244, Asp307, and Arg310 (Palazzotti et al., 2019). These residues line the central cavity and form hydrophobic, aromatic, and electrostatic interactions with substrates and inhibitors (Yu et al., 2022; Brawley et al., 2022; Palazzotti et al., 2019; Işık & Serçinoğlu, 2025).

To increase bacterial susceptibility to antibiotics, many compounds have been proposed as inhibitors of efflux pumps. These compounds have not been able to meet clinical and therapeutic standards because their adverse toxicity profile (Zimmermann et al., 2019). The goal of developing novel efflux pump inhibitors is to achieve synergistic antibacterial activity (Zimmermann et al., 2019). A huge number of potential efflux pump inhibitors, including natural compounds and synthetic compounds, have been tested to evaluate their efficacy and toxicity (Wang et al., 2016). Kumar & Tudu, (2023) reported that experimental results revealed the combined activity of capsaicin (an analogue of a plant-derived bioactive compound) and ciprofloxacin reduced the minimum inhibitory concentration (MIC) of ciprofloxacin from two- to four fold. Reserpine is a plant-derived alkaloid that has widely been

studied both in medicine and microbiology. It is derived from *Rauwolfia serpentine* (Indian snakeroot). Its pharmacological relevance include antihypertension (Siddiqui et al., 2020; Weir, 2020), antipsychotic (Nur & Adams, 2016; Carlsson, 1975), and in microbiology as an efflux pump inhibitor. Reserpine significantly reduced the MIC and IC<sub>50</sub> of selected antibiotics by up to four-fold (Guedes et al., 2014). Reserpine, although effective, has some serious side effects (Strawbridge et al., 2023; Sarwer-Foner et al., 1956). Reported side effects include depression and neurotoxicity (Govindarajulu et al., 2021; Salimikia & Heidari, 2023; Strawbridge et al., 2023). However, reserpine is still widely used as a reference inhibitor for *S. aureus* NorA efflux pump.

Molecular docking helps to predict the behavior of a ligand or potential drug candidate in the binding pocket or active site of a macromolecule such as protein (Paggi et al., 2024). It shows the orientation and position of the ligand in the pocket of protein (Muhammed & Aki-Yalcin, 2024). Moreover, it facilitates our understanding of the different molecular interactions or forces that are necessary for the stability of a ligand in the binding pocket of the protein. Through this understanding, effective and novel drugs can be effectively designed and maximized (Morris et al., 2009). Structural biology techniques including X-ray crystallography, NMR spectroscopy, and cryo-EM (Electron Microscope) reveal the atomic details of protein structures, including the active sites and binding pockets, which provides an important framework for drug design and development (Li et al., 2022). By integrating experimental structures with computational tools, researchers can visualize key residues involved in ligand recognition and anchorage. This insight allows researchers to design or screen compounds that can competitively or allosterically inhibit the target protein (de Araújo et al., 2021).

This study aims to uncover the molecular determinants (bonds, chemical groups, and amino acids) responsible for the stabilization of reserpine in the pocket of NorA efflux pump. This study will provide mechanistic insight into the molecular interaction between reserpine and the residues in the substrate-binding pocket of NorA efflux pump. By uncovering these molecular interactions, novel drugs will be

designed to maximize these ligand-stabilizing interactions while effectively reducing their adverse effects. Efficient stabilization of the ligand is mainly achieved through strong hydrogen bonding and hydrophobic contacts; hydrogen bonds ensure binding affinity and specific spatial orientation, while hydrophobic contacts further enhance affinity through water displacement and the subsequent gain in entropy (Klebe, 2025).

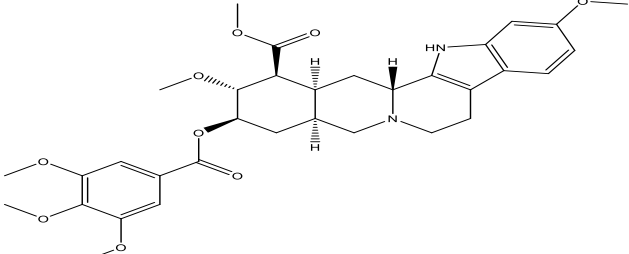
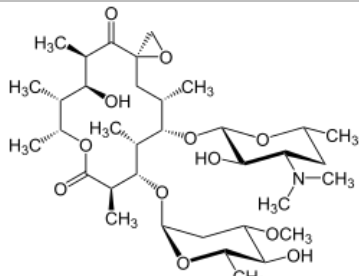
## Method

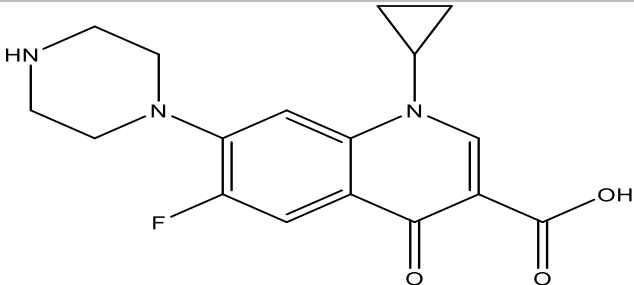
### Ligand Retrieval and Preparation

The three-dimensional (3D) conformation of the ligand molecules were obtained and processed according to the following procedure. All

compounds listed in **Table 1** were obtained from the PubChem database (Morris et al., 2009). The 3D molecular structures of reserpine (CID: 5770), oleandomycin (CID: 72493), and ciprofloxacin (CID: 2764) were downloaded in SDF file format and subsequently imported into Avogadro software (version 1.2.0) for explicit hydrogen addition and energy minimization to resolve structural constraints (Hanwell et al., 2012). Following energy optimization, the ligands were exported in PDB format and transferred into AutoDockTools (ADT) (v. 1.5.7) for the assignment of Gasteiger partial charges and definition of rotatable bonds (Ferreira et al. 2015). The prepared ligands were ultimately saved in PDBQT file format to complete ligand preparation for docking.

**Table 1.** 2D Structures of Ligands

Ligand	Molecular Weight (g/mol)	2D Structure
Reserpine	608.7	
Oleandomycin	687.9	

Ciprofloxacin	331.34	
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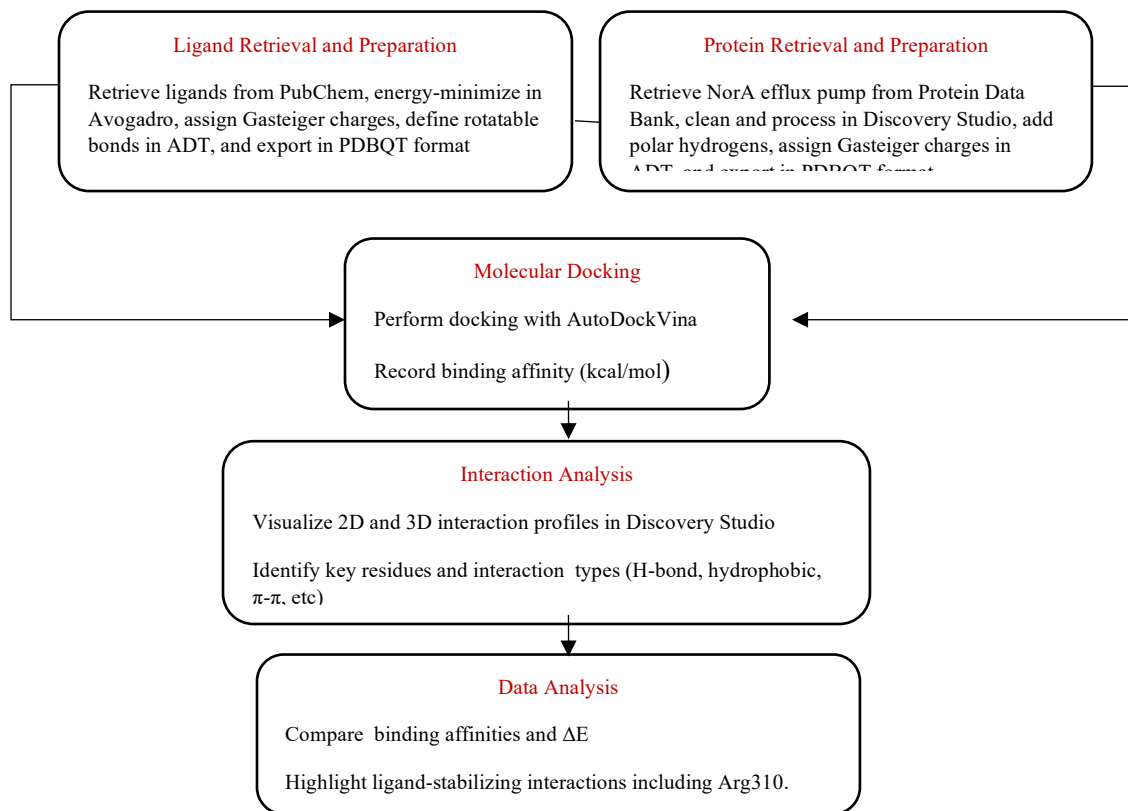
#### *Protein Retrieval and Preparation*

The NorA efflux pump protein of *Staphylococcus aureus* (PDB ID: 7LO8) was obtained from the Protein Data Bank. The structure was processed in BIOVIA Discovery Studio 2025 (DassaultSystèmes) for initial cleaning. Following this step, the protein was exported and prepared in AutoDockTools, where polar hydrogens were introduced, Gasteiger charges were assigned, and the final structure was converted to PDBQT (Protein Data Bank, Partial Charge (Q), and Atom Type (T)) format for docking (Swamy et al., 1997).

#### *Molecular Docking and Data Analysis*

Molecular docking was conducted with AutoDockVina, an open-source docking tool designed to predict the binding orientation and affinity of small molecules (ligands) to a specific target protein (receptor).

In this investigation, the docking outcomes were assessed according to two key criteria: the binding affinity (represented in kcal/mol) and the interaction profile of the ligand with the residues of the target protein. Binding affinity offers an approximation of the potency of the ligand-protein complex, whereas the interaction profile delineates the particular non-covalent forces, including hydrogen bonds, hydrophobic contacts,  $\pi$ - $\pi$  stacking, and electrostatic interactions, that facilitate binding. In this study, data analysis prioritized the most pharmacologically and biologically relevant binding affinity, which is based on engagement with critical residues and interaction types, notably conventional hydrogen bonds due to their directional nature and considerable strength (1–7 kcal/mol). Hydrophobic contacts and other stabilizing interactions, such as van der Waals forces,  $\pi$  interactions and carbon hydrogen bonds, were also taken into account.



**Fig 1.** Schematic Representation of the Molecular Docking Workflow

## Results

We reported the energy difference ( $\Delta E$ ) between the two top poses in order to improve the reliability of our docking results. When the energy difference (**Table 2**) is very small ( $\Delta E \leq$

1 kcal/mol), it means the two poses are almost equally stable. The most pharmacological relevant pose will be highlighted because it represents the biologically plausible interaction and conformation of the ligand in the pocket of the target protein.

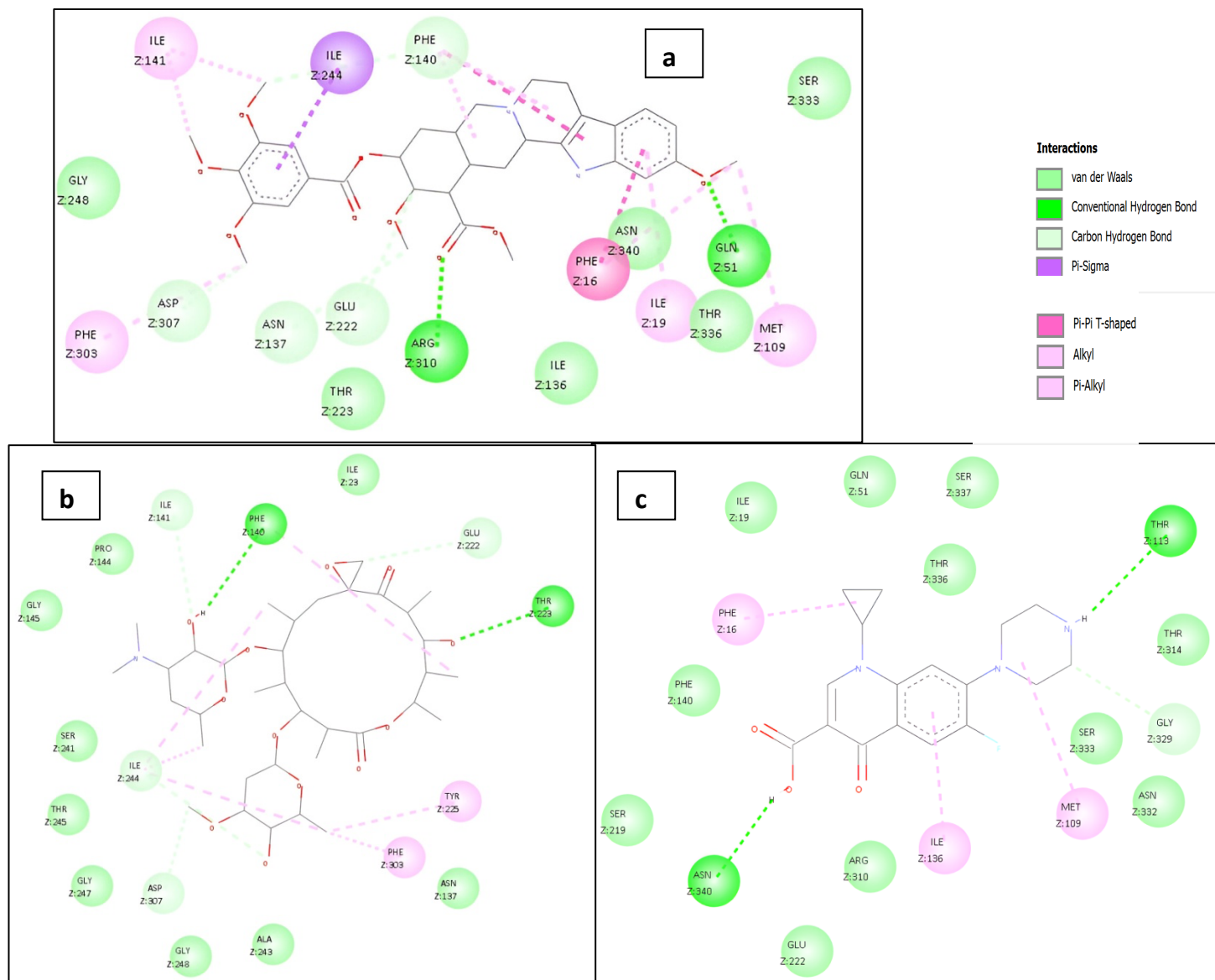
**Table 2.** Top Two Binding Affinities and Energy Difference, and Key Molecular Interactions

Ligand	Pose 1 (kcal/mol)	Pose 2 (kcal/mol)	$\Delta E$ (kcal/mol)	Interactions
Reserpine	-9.5*	-9.2	-0.3	Conventional hydrogen bond, van der Waals forces, carbon hydrogen bond, pi-sigma, pi-pi T-shaped, alkyl.
Oleandomycin	-8.9	-8.6*	-0.3	Conventional hydrogen bond, van der Waals, carbon hydrogen bond, alkyl.
Ciprofloxacin	-8.3*	-7.8	-0.5	Conventional hydrogen bond, van der Waals, carbon hydrogen bond, alkyl.

An asterisk (\*) indicate the most pharmacologically relevant pose based on binding affinity and interaction profile.

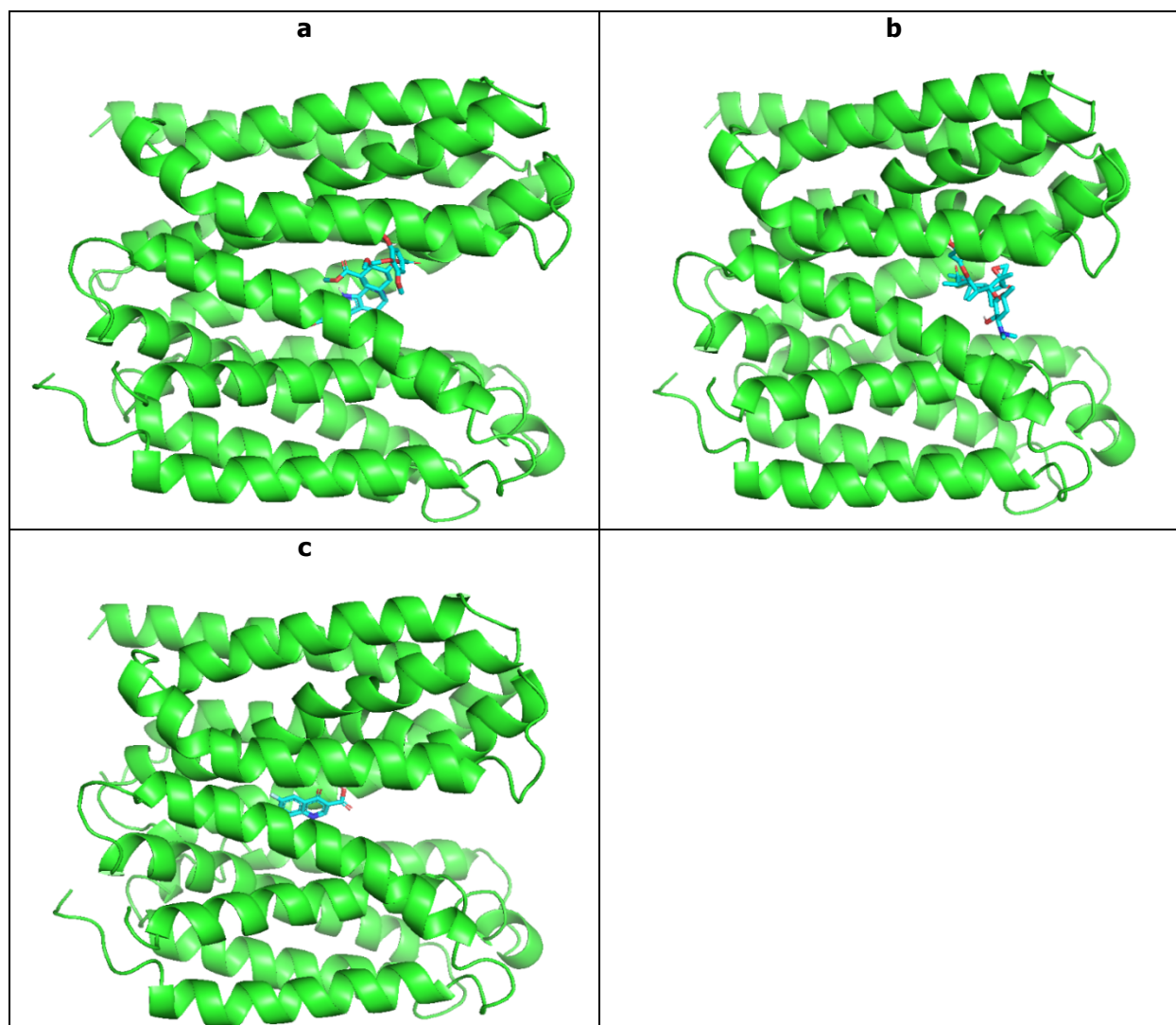
BIOVIA Discovery Studio 2025 was used to visualize and generate the two-dimensional (2D) (Figure 1) docking poses below, which represent the most pharmacologically and biological relevant poses for each ligand. These

poses are analyzed to evaluate the specific nature of the ligand-protein interactions, with a specific focus on conventional hydrogen bonding.



**Fig 2.** 2D interaction profile of ofreserpine (a), oleandomycin (b), and ciprofloxacin (c) within the substrate-binding pocket of NorA efflux pump.

The three-dimensional (3D) (Figure 3) poses below represents the most pharmacologically and biologically relevant poses for each ligand. The 3D ribbon images below provides insight into the spatial orientation of the ligands in the binding pocket of NorA.



**Fig 3.** Reserpine (a), oleandomycin (b), and ciprofloxacin (c) in the binding pocket of NorA efflux pump

### Discussion

Molecular docking analysis offers valuable information regarding the binding affinity and molecular interactions between a protein and a ligand. This information is crucial for the discovery and development of new pharmaceutical compounds (Guedes et al., 2014). In docking studies, more negative numerical values indicate a stronger binding affinity (Saikia & Bordoloi, 2019). Consequently, according to the docking results, the ligand possessing the greatest binding affinity is reserpine (-9.5 kcal/mol). This high binding affinity for reserpine is supported by an earlier docking study that demonstrated reserpine binds to the *S. aureus* NorA efflux pump with an

affinity of -9.4 kcal/mol (Zimmermann et al., 2019). The marginal difference of -0.1 kcal/mol may be attributed to differences in docking protocol/methodology. Such methodological factors can be attributed to variations in protein model (experimental vs. homology-modelled). Other contributing factors may include variations in docking software versions, grid box dimensions, exhaustiveness, protonation, charge assignment, treatment of rotatable bonds, crystal protein quality, ligand and protein preparation. However, both scores (-9.5 and -9.4 kcal/mol) provide sufficient evidence that reserpine exhibits high-affinity binding to the substrate-binding pocket of NorA. NorA's primary role in MDR is to confer resistance to the

bacterial cell by expelling hydrophilic fluoroquinolones such as ciprofloxacin (Yu et al., 2022; Salih, 2022).

About 65% of residues in the substrate-binding pocket of NorA are hydrophobic residues (Brawley et al., 2022). Also, this substrate-binding pocket is rich in aromatic amino acids which include Phe 16, Phe 140, Phe 303, and Phe 306; these aromatic residues may be essential for the binding of aromatic drugs such as fluoroquinolones (of which ciprofloxacin is a member) (Brawley et al., 2022). Notably, Glu 222 and Asp 307 play a very crucial role in the functional mechanism of the NorA efflux pump (Brawley et al., 2022; Li et al., 2024). Their essential role in the substrate-binding domain is largely due to their ionizability. These acidic amino acids (Glu 222 and Asp 307), located in the substrate-binding domain, are largely responsible for substrate-binding because they create an anionic patch which effectively attracts positively charged substrates such as ciprofloxacin. The protonation – deprotonation dynamic state of these acidic residues is essential for trapping and expelling substrates including antibiotics (Brawley et al., 2022; Li et al., 2024). Mutation of these amino acids abolished the function the protein (Brawley et al., 2022). In addition, reserpine interacted with other key residues, including Phe 140 and Ile 244, which may also be involved in substrate recognition and binding (Yu et al., 2022; Brawley et al., 2022; Palazzotti et al., 2019; Işık & Serçinoğlu, 2025).

In the 2D interaction profile, reserpine made interactions with Glu 222 and Asp 307 through carbon-hydrogen bond (a form of electrostatic interaction). Although, a weak bond, it may significantly contributed the high-binding affinity and inhibitory potential of reserpine. Key aromatic residues such as Phe 16, Phe 140, and Phe 303, along with the ionizable residue Arg 310, play a critical role in ligand binding and stabilization (Brawley et al., 2022). Reserpine formed a conventional hydrogen with Arg 310, which may be very essential for the stabilization of the ligand. This is in agreement with Rodrigues et al. (2022), who reported that khelin and visnagin, in a molecular docking study, formed hydrogen bond with Arg 310, a critical residue in the substrate-binding pocket of NorA. Khelin and visnagin were investigated as

potential EPIs, and their strong interaction with Arg 310 supported their potentials as EPIs (Rodrigues et al., 2022). Ciprofloxacin did not exhibit strong interaction with Arg 310, which may have impacted binding affinity. Co-administering EPIs with antibiotics can significantly enhance antimicrobial activities, which is evidenced by significant reduction in MICs and IC50s (Schmitz et al., 1998). Reserpine increased bacteria susceptibility to moxifloxacin, sparfloxacin and ciprofloxacin (Schmitz et al., 1998; Huang et al., 2013). In a docking experiment involving acriflavine resistance protein B (AcrB) (another type of efflux pump especially found in gram negative bacteria), docking results suggests that reserpine shares binding site with ciprofloxacin; hence, reserpine can potentially inhibit ciprofloxacin from binding through competitive inhibition (Shaheen et al., 2019). Another important point to note is that, in addition to molecular interactions, the structure and spatial orientation (**Figure 2**) of the ligand also impacts binding affinity (Swamy et al., 1997).

## Conclusion

This study successfully elucidated the molecular determinants underpinning the high-affinity binding of reserpine to the *Staphylococcus aureus* NorA efflux pump. Reserpine demonstrated superior binding affinity (-9.5 kcal/mol) and formed critical interactions with key residues (Glu222, Asp307, and Arg310) in the hydrophobic substrate-binding pocket, highlighting its potent inhibitory potential. These insights provide a structural blueprint for designing novel, targeted efflux pump inhibitors that maximize stabilizing interactions while minimizing adverse effects. Targeted introduction of hydrogen bond donors or a protonable amine directed at the carboxylate (COO<sup>-</sup>) side chain of Glu 222 and Asp 307 may convert weak carbon hydrogen (C-H) bond into stronger, directional interactions and effectively increase NorA inhibition. Most importantly, any gains in potency should be evaluated alongside ADMET parameters to avoid exacerbating the known toxicological limitations of reserpine. Elucidating key interactions (bonds and residues) that confer high-affinity binding to NorA's pocket, is important for designing more potent and safer EPIs.



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