Glycosylated Antibiotics: New Promising Bacterial Efflux Pumps Inhibitors

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Abstract

Antimicrobial resistance is considered a major concern; bacteria have evolved mechanisms to overcome antibiotics' action through evolutionary process. One major resistance mechanism that bacteria developed is the pumping of the antibiotics out of bacterial cells by transmembrane transporter proteins known as efflux pumps. To overcome such resistance, small molecules known as efflux pumps inhibitors (EPIs), are created to inhibit the efflux pumps functions. The new inhibitors are glycosylated antibiotics which have been created by adding N-acetyl glucose amine moiety to acceptor hydroxyl group of five common antibiotics are Ampicillin, Amoxicillin, Chloramphenicol, Ciprofloxacin, and Tetracycline. To test the effectiveness of the new EPIs in inhibiting AcrB-TolC and MexA-OprM efflux pumps functions, docking and simulation studies have been applied. Docked glycosylated tetracycline has given the highest binding energy in the active sites of both pumps, with -9.4 against AcrB and -8.8 against MexA. The simulation study has confirmed the binding of the glycosylated tetracycline in the active sites of both pumps, as well as its stability during the biological dynamicity of both pumps (opening and closing channels). However, for the cost purpose, this study included short simulation time of 2ns that prevent the proteins-ligands complexes from reaching the plateau.

Key words

Antibiotics; Antimicrobial resistance; Molecular docking; Efflux pumps inhibitors; Glycosylated antibiotics

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82 Introduction:
83 Bacterial antibiotics resistance is a globally concern problem, bacteria have evolved mechanisms
84 to counter antibiotics' actions, main one involving the pumping out of antibiotics from bacterial
85 inside into the external environment by specialized membrane proteins known as efflux pumps,
86 which confer bacterial resistance to a wide range of antibiotics [1,2]. Efflux pumps are bacterial
87 transport proteins that extrude toxins and antibiotics out of bacterial cells by energy dependent
88 mechanisms [3], thereby classified into ABC active transporters, derived their energy from ATP
89 molecules while other transporters' families derived their energy by chemical gradients which are
90 MFS (Major Facilitator Superfamily), MATE (Multidrug and toxic compounds extrusion), SMR
91 (The small multidrug resistance), RND (Resistance Nodulation-division), and DMT (Drug
92 metabolite transporter) [4,5]. Well studied efflux pumps are AcrB-TolC in *E.coli* and Mex-OPrM
93 that mostly found in *Pseudomonas aeruginosa* [6,7], molecular based structures for both
94 transporters have been elucidated extensively, TolC and OprM are tunnel structures that span the
95 outer membrane of Gram-negative bacteria, It's a homotrimer composed of α- helical barrel, β-
96 barrel and α/β mixed fold [1], AcrB and Mex are fusion or linker proteins that are directly in
97 contact with TolC and OprM respectively, whereas the inner cytoplasmic domain is the transporter
98 protein, some of its parts spanning the cytoplasmic membrane to come in contact with fusion parts,
99 for better descriptions, both pumps structures are illustrated in **(Figure 1)**, [1,8].
100 As the important role of bacterial efflux pumps in bacterial treatments' failures and concurrent
101 bacterial infections, numerous natural and synthetic compounds have been repurposed as efflux
102 pumps inhibitors (EPIs), such compounds either cause conformational change in the structure of
103 efflux pumps to inhibit its functional assembly or block the substrates binding site of the efflux
104 pumps [9]. PAβN (Phenylalanine-arginine β-naphthylamide) is the first discovered inhibitor for
105 Mex-OprM efflux pump that blocks the binding site of the pump resulting in an increase of the
106 efflux sensitive antibiotic inside bacterial cells, Pyridopyrimidine derivative D13-9001 restore the
107 antibiotic activity by competitive binding with the substrate binding site of AcrB-TolC and Mex-
108 OprM efflux pumps [10]. Several other natural EPIs are plants alkaloids, polyphenols, quinolones
109 derivatives and others, majority of them with unknown exact mechanisms of action [4,11].
110

EPIs are considered as potential antibiotics adjuncts to overcome multi-drugs resistance, working by combining EPIs with antibiotics course of treatments, or simply just modifying the antibiotics structure to create new EPIs or so called antibiotics analogues such as tetracycline analogue that recently known to restore tetracycline effectiveness in resistant bacteria [12], glycosylation of antibiotics is mostly common among macrolides, bacterial glycosyltransferase transfers UDP-glucose moiety into antibiotic's OH receptor and circumvents the antibiotics activity [13,14]. Glycosylated antibiotic can be synthesized through modifying the chemical structure of the specified antibiotic, such new class of antibiotics might restore the antibiotic activity in the resistant bacteria by working as EPIs or antibiotics analogues, to test the hypothesis, sweet antibiotics have been docked against the active binding site of AcrB and MexA efflux pumps and molecular dynamic simulation has been applied to figure out the conformational changes of the mentioned efflux pumps upon sweet antibiotics binding.

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152 **Results**
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154 **1. Pharmacokinetic properties**
155 Lead likeness properties for all five glycosylated antibiotics were measured, Lipinski's role of 5 to
156 predict drugs likeness properties for all new glycosylated antibiotics were followed, the drugs
157 criteria are MW of the lead $\leq$ 500, less than 5 hydrogen donors, less than 10 hydrogen bond
158 acceptor, Log P or lipophilicity not greater than 5 [15], all lead-likeness properties along with
159 Lipinski's number of violations are listed in (**Table 1)**.
160
161 **2. Docking results**
162 The binding energy of all ligands "glycosylated and non-glycosylated antibiotics" against AcrB and
163 MexA efflux pumps are illustrated in (**Tables 2, 3)**. Glycosylated tetracycline has given the highest
164 binding energy against both efflux pumps, with -9.7 against AcrB efflux pump and -8.8 against
165 MexA, the interacted amino acids residues in the binding pockets for both pumps against
166 glycosylated tetracycline are explained in **(Figures 2, 3)**. Glycosylated tetracycline has shown
167 some similarity in the binding pocket of MexA efflux pump when compare it to the binding pocket
168 pyridopyrimidine in the same pump, shared amino acids residues are GLN110, LYS 108 and SER
169 107 **(Figure 3b)**. of Two EPIs were included in the docking against studied efflux pumps for
170 comparison.
171
172 *3. Molecular dynamic simulation*
173 RMSD value for the 6TA6- glycosylated tetracycline complex was plotted as in **(Figure 4)**, protein-
174 ligand complex was not reaching the plateau since short time of simulation was devoted.
175 However, the efflux pump dynamicity has been captured during the opening and the closing state
176 of the pump **(Figure 5)**. The md simulation of AcrB efflux pump was done in 2 ns scale, RMSD
177 value for AcrB-glycosylated tetracycline complex is explained in **(Figure 6)**, whereas **(Figure 7)**
178 shows the complex conformational change during the opening and the closing process.
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Discussion

In order for the newly drugs candidates to pass clinical trials, there are set of ADME properties that the drugs should meet. ADME stands for Absorption, Distribution, Metabolism and excretion, most of drugs are administered orally, so the oral bioavailability is such an important feature [16].

Three out of the five glycosylated antibiotics in the current study were seen to violate Lipinski's rule for molecular weight determinant. As the importance of low molecular weight for orally administered drugs [17], however, there are other important features that all the understudied glycosylated antibiotics have such as acceptable oral bioavailability, no toxicity and no blood brain barrier permeability. A study has confirmed the presence of many FDA approved antibiotics and antifungals that have violated the rule of 5, the same study had explained that such drugs are still orally bioavailable because it has a specific group that works as transporters' substrate [18]. The rule of 5 appears as important data set that the oral rout drugs should meet, in a study has examined about 111 non-oral rout drugs, concluded that Lipinski's criteria for such drugs are not so important to meet [19].

The drugs' journey starts by its absorption through gastrointestinal system, reaching blood stream and binding with the specified target to exert its action. The targeted proteins are two bacterial efflux pumps known as AcrB and MexA, the glycosylated tetracycline has shown the highest binding energy against both target proteins. Tetracycline resistance mediated by efflux pumps has increased recently, various factors are responsible for such resistance rise including the pumps genes are DNA mobilized that can transfer from one bacterium to another. Another factor is the long-term use of tetracycline causing the bacteria to co- evolve itself to extrude such toxins outside of the bacterial cells [20]. Developing molecules that target bacterial efflux pumps has been considered as a new treatment approach, such molecules are known as efflux pumps inhibitors (EPIs). One of the big issues that hurdle the use of EPIs is their toxicity to human cells [20]. Finding safe and effective EPIs is on the increase, of which one potential way is through modifying the structure of the effluxed antibiotics, such modification might help in blocking the binding capability of the used antibiotic and successfully reverse the antibiotic resistance mechanism [21]. New tetracycline derivative known as glycylcycline has been developed as a combination therapy along with tetracycline to overcome Tet efflux pump resistance [22]. Glycylcycline which sometimes known as a tigecycline has similar structure to tetracycline with substitution of an N-alkylglycylamido group at D-9 position [23]. Another modification can be achieved through adding various glycosidic chains at C, O, N atoms on antibiotics, such glycosylation is a naturally occurring process that have been observed among number of

antibiotics including macrolides and streptomycin [24]. Understudied glycosylated antibiotics have shown promising binding energy against the target proteins in compare to synthetically available EPIs. Because the targets are efflux pumps transporter proteins which are a dynamic structure, molecular dynamic simulation has been applied to test the stability of the newly drugs in the binding pockets of its targets.

The molecular dynamic simulation studies of bacterial efflux pumps are a new era of interesting, investigating ligands stability among different conformational changes that efflux pumps undergone such as the transition from substrate binding state into opening state is facing issues at large scale proteins level [25]. The first md simulation studies for AcrB-TolC and MexA-OprM have been started since 1999 and still ongoing progresses till now, different simulation approaches have been used including mutable basin simulation, steered md simulation and un biased equilibrium md simulation [26]. Identifying ligand binding site or what's so called the hotspots is crucial in drug discovery, mixed solvent md simulation is successfully used for such a purpose, one of its big limitation is the cost of running such kind of simulation [27].

Conclusion: Glycosylated tetracycline is showing promising results to work as EPIs without worrying about safety issues, however, it's clinical approving require applying longer simulation time of 50 ns or more, as well as further in vitro studies are required to measure its activity against resistant bacteria. Glycosylation of other antibiotics with lower molecular weight and less Lipinski's role of 5 violations are recommended.

Materials and Methods

1. Glycosylation of antibiotics

Three dimensional structures of amoxicillin, ampicillin, chloramphenicol, ciprofloxacin and tetracycline were retrieved from pubchem web server (https://pubchem.ncbi.nlm.nih.gov/) in simple document format (SDF). The Pubchem CIDs for the studied antibiotics are 33613, 6249, 5959, 2764, and 54675776 respectively.

N-acetyl glucose amine moiety has been added to the hydroxyl acceptor group for each selected antibiotic, using Marvin sketch software 20.20, then the ligands were energetically optimized to the most stable structures using merk molecular force field 94 (MMFF94).

2. Pharmacophore kinetics properties

The drug-likeness properties have been measured for all glycosylated antibiotics using the website http://www.swissadme.ch/. The role of 5 for Lipinski as well as others features have been measured including the oral bioavailability, GI absorption, Blood brain barrier permeability, and liver cytotoxicity.

3. Molecular docking

3D x-ray crystallographic structures of AcrB and MexA were retrieved from the protein data bank (PDB) with IDs 2I6W and 6TA6 respectively. After which the proteins were prepared for docking and protein-ligand complexes minimized using the relevant tools in Cresset Flare© software, version 4.0 (https://www.cresset-group.com/flare/). The protein minimization was done based on the GAFF force field, with gradient cutoff of 0.200 kcal/mol/A and iterations was set to 2000 iterations [28]. Furthermore, the docking was achieved using flexible docking protocol [29]. In brief; Python Prescription 0.8, a suite housing Auto Dock Vina, was employed for the molecular docking study of the selected ligands with the two efflux pumps. The specific target site for AcrB was set using the grid box with dimensions (29.1800 x 22.3491 x 31.3636) Å, while that of MexA was (68.1632 x 57.6364 x 103.9827) Å and their centres were adjusted based on the active site of the enzymes. At the end of the molecular docking, 10 binding poses with least binding affinity for each protein-ligand complex were generated for all the ligands, scoring results were also created after retrieving from text files which were employed for comparative analysis. The protein-ligand complexes as well the molecular interactions were all visualized in 3D and 2D using PyMOL© Molecular Graphics (version 2.4, 2016, Shrodinger LLC) and Protein- Ligand interaction Profiler PLIP server (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index).

4. Molecular dynamic simulation

For glycosylated tetracycline which give the lowest binding affinity against both efflux pumps' binding sites, molecular dynamic simulation was performed using NAMD 2.8, the topology file for the efflux pumps proteins were generated using automatic PSF builder in VMD program, whereas, the topology file for the ligand was generated using CHARMM GUI server (http://www.charmm-gui.org/), 2ns simulation was performed with time step of 2 fs, periodic boundary conditions were used, the temperature kept at 310 K and the pressure was stable at 1.013 bar, 100 steps of equilibration were completed before starting the solvate simulation, the whole md simulation protocol can be found [30]. CDC trajectory file for both efflux pumps- ligand complexes was analyzed in the form of measuring RMSD values using VMD graphical visualization program 1.9 version.

Authors' contributions

ZK carried out the study experimental design, molecular dynamic simulation work and writing the first draft of the research paper. HU carried out the modification of antibiotics' structures work, did part of the docking work and reviewed the paper. PD did most of the docking work, and analyzed the docking results. PS confirmed the docking results by replicated it and reviewed the paper.

Competing interests

The authors have declared no competing interests

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349 **References**
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437 ## Figure legends
438
439 **Figure 1 The structural assembly of MexA efflux pump**
440 The structural components of MexA efflux pump on the left panel, that its similar to the structure
441 of AcrB efflux pump on the right side, the Image has been taken from [1].
442
443 **Figure 2 The binding site of the glycosylated tetracycline in Acrb efflux pump binding pocket**
444 **A.** The left side is the binding pose of the glycosylated tetracycline
445 **B.** The right side represents the interaction between amino acids residues of AcrB chain A and
446 the glycosylated tetracycline, the new antibiotic showed hydrophobic interaction with PRO
447 40A, GLU 673A and Hydrogen bonds with TYR 77A, SER 79A, THR 91A, SER 134A,
448 GLU 683A, GLU 826A.
449
450 **Figure 3 The binding sites of all of the glycosylated antibiotics in the binding pocket of MexA**
451 **efflux pump**
452 **A.** The left side, shows the binding pose of amoxicillin (cyan), ampicillin (pink), chloramphenicol
453 (yellow), ciprofloxacin (red) and tetracycline (blue) in MexA. The drugs occupy similar region in
454 MexA except amoxicillin.
455 **B.** The right side is the interaction between residue of MexAB – OprM efflux pump and
456 Glycosylated tetracycline, that showed hydrophobic interaction ASP 230D, THR 233D, GLU 796J
457 and hydrogen bonds with PRO 32D, ASN 36D, ARG 147E, ASP 176D, THR 178D, ASN 194J,
458 SER 235D, VAL 236D, THR 237D, MET 797.
459
460 **Figure 4 The binding site of pyridopyrimidine derivative D13-9001**
461 Interacted amino acids residues in the binding pocket of MexA upon Pyridopyrimidine binding,
462 shared residues with the glycosylated tetracycline binding pocket are highlighted in yellow.
463
464 **Figure 5 RMSD analysis for MexA-OprM- glycosylated tetracycline complex**
465 CDC trajectory file for the solvate complex was used to analyze the RMSD value, 1150 frames
466 were used, the protein-ligand complex didn't reach the plateau because of short simulation
467 time.
468
469 **Figure 6 MexA efflux pump opening and closing states upon glycosylated tetracycline binding**
470 the dynamic movement of MexA efflux pump upon binding with the glycosylated tetracycline, it
471 shows the stability of the ligand during the closing of the channel **A.** and the opening of the
472 channel **B.**

Figure 7 RMSD analysis for AcrB- glycosylated tetracycline complex

RMSD value for AcrB- glycosylated tetracycline was calculated using VMD program, 4250 frames were being used in the analysis of the CDC trajectory file. Again protein-ligand complex didn't reach the plateau.

Figure 8 AcrB efflux pump opening and closing state upon glycosylated tetracycline binding

Shows the closing state **A.** and the opening of AcrB efflux pump **B.**, the glycosylated tetracycline is stable in the binding pocket of the target protein.

Tables

Table 1 Pharmacokinetics properties of the newly glycosylated antibiotics.

Table 2 Docking results of N-acetyl glucose amine antibiotics against AcrB efflux pump in *E. coli* and MexA efflux pump in *Pseudomonas aeruginosa*.

Table 3 Binding Energy of Non- glycosylated antibiotics docked against ArcB and MexA.

Supplementary material

Figure 9 The binding sites of all glycosylated antibiotics against AcrB efflux pump

Binding pose of amoxicillin (yellow), ampicillin (red), chloramphenicol (white), ciprofloxacin (blue) and tetracycline (orange) in AcrB. The drugs occupy similar region in AcrB.

Figure 10 The binding sites of all glycosylated antibiotics against MexA efflux pump

Binding pose of amoxicillin (cyan), ampicillin (pink), chloramphenicol (yellow), ciprofloxacin (red) and tetracycline (blue) in MexA. The drugs occupy similar region in MexA except amoxicillin.

Table 4 The binding affinities of the glycosylated tetracycline against AcrB and MexA.

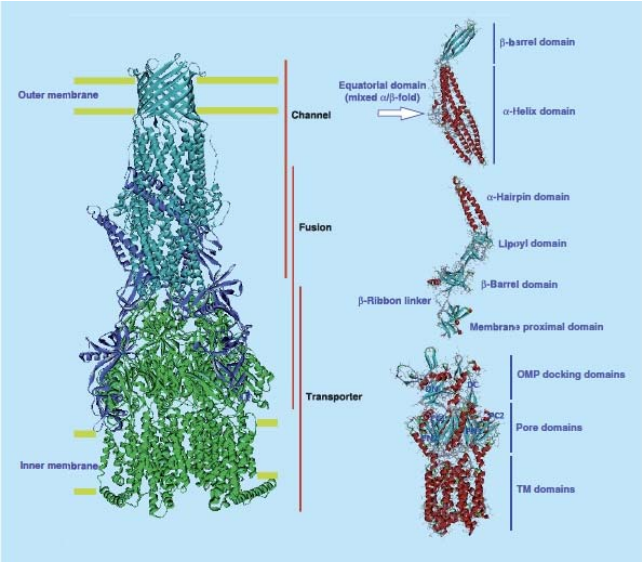

Figure 1 The structural assembly of MexA efflux pump

The structural components of MexA efflux pump on the left panel, that its similar to the structure of AcrB efflux pump on the right side, the Image has been taken from [1].

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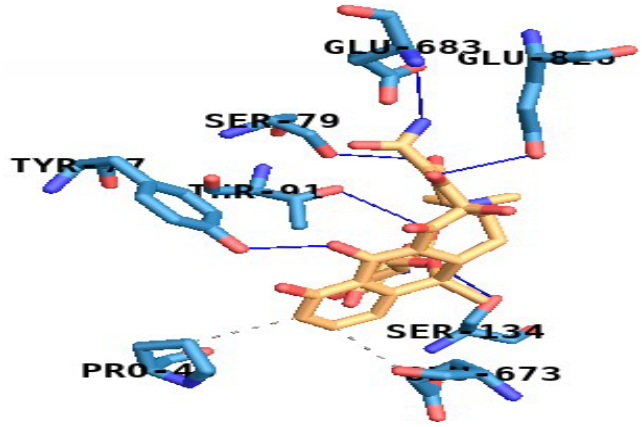
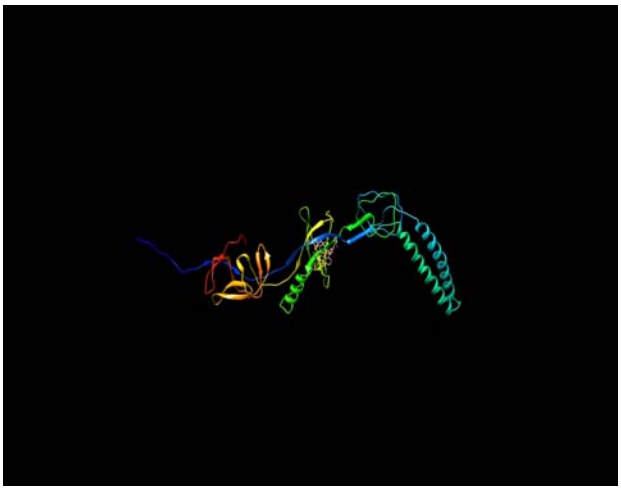


B

Figure 2 The binding site of the glycosylated tetracycline in Acrb efflux pump binding pocket

A. The left side is the binding pose of the glycosylated tetracycline

B. The right side represents the interaction between amino acids residues of AcrB chain A and the glycosylated tetracycline, the new antibiotic showed hydrophobic interaction with PRO 40A, GLU 673A and Hydrogen bonds with TYR 77A, SER 79A, THR 91A, SER 134A, GLU 683A, GLU 826A.



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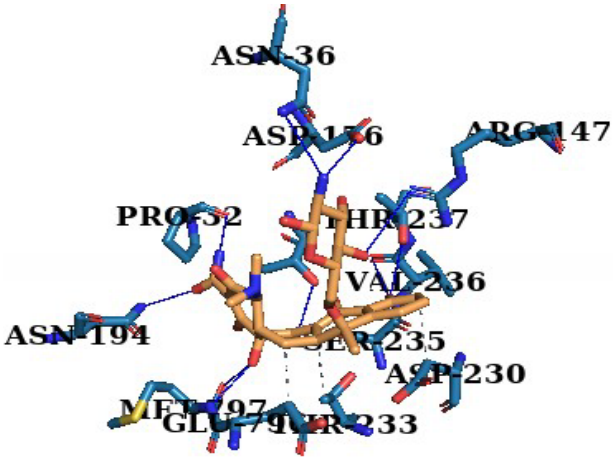

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Figure 3 The binding sites of all of the glycosylated antibiotics in the binding pocket of MexA efflux pump

A. The left side, shows the binding pose of amoxicillin (cyan), ampicillin (pink), chloramphenicol (yellow), ciprofloxacin (red) and tetracycline (blue) in MexA. The drugs occupy similar region in MexA except amoxicillin.

B. The right side is the interaction between residue of MexAB – OprM efflux pump and Glycosylated tetracycline, that showed hydrophobic interaction ASP 230D, THR 233D, GLU 796J and hydrogen bonds with PRO 32D, ASN 36D, ARG 147E, ASP 176D, THR 178D, ASN 194J, SER 235D, VAL 236D, THR 237D, MET 797.

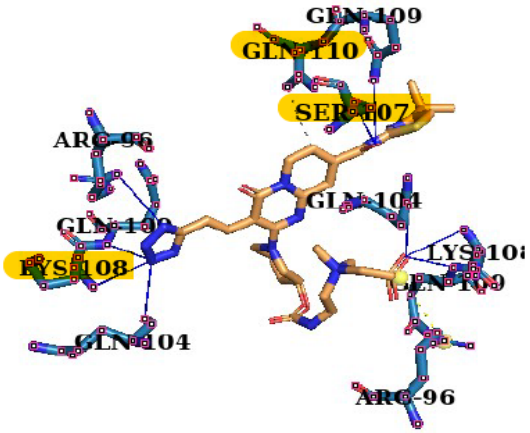


Figure 4 The binding site of pyridopyrimidine derivative D13-9001

Interacted amino acids residues in the binding pocket of MexA upon Pyridopyrimidine binding, shared residues with the glycosylated tetracycline binding pocket are highlighted in yellow.

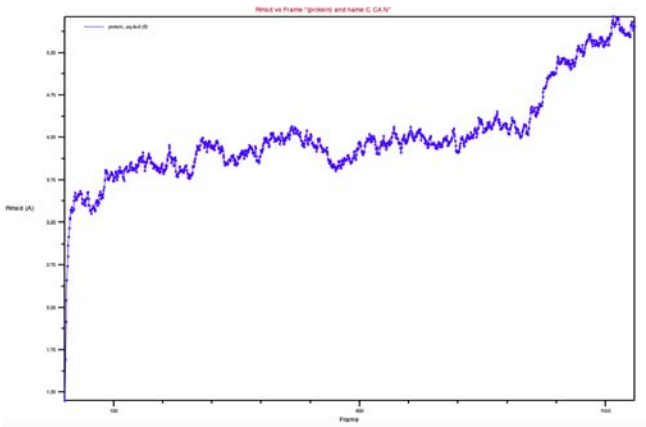
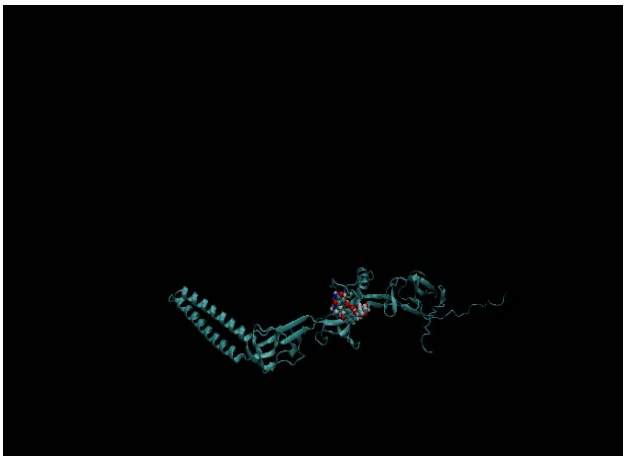


Figure 5 RMSD analysis for MexA-OprM- glycosylated tetracycline complex
CDC trajectory file for the solvate complex was used to analyze the RMSD value, 1150 frames were used, the protein-ligand complex didn't reach the plateau because of short simulation time.



A

B

Figure 6 MexA efflux pump opening and closing states upon glycosylated tetracycline binding
the dynamic movement of MexA efflux pump upon binding with the glycosylated tetracycline, it shows the stability of the ligand during the closing of the channel **A.** and the opening of the channel **B**.

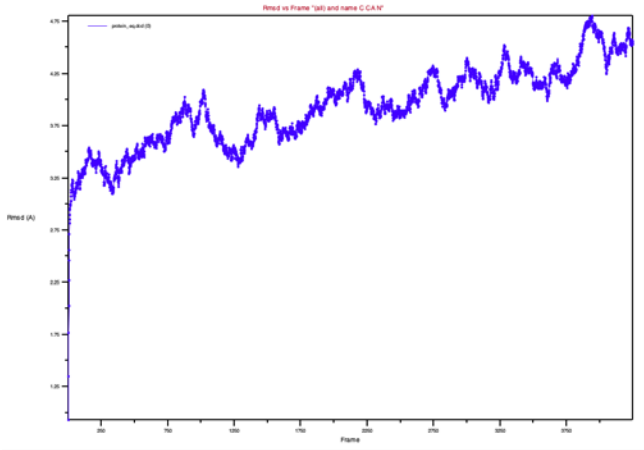
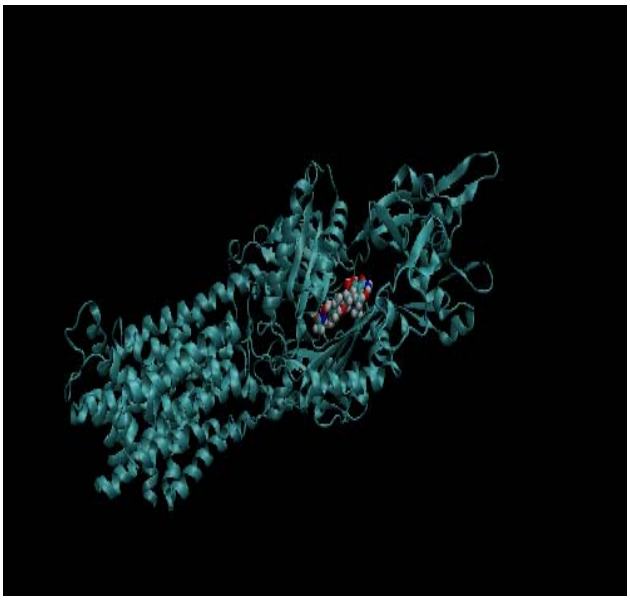


Figure 7 RMSD analysis for AcrB- glycosylated tetracycline complex

RMSD value for AcrB- glycosylated tetracycline was calculated using VMD program, 4250 frames were being used in the analysis of the CDC trajectory file. Again protein-ligand complex didn't reach the plateau.



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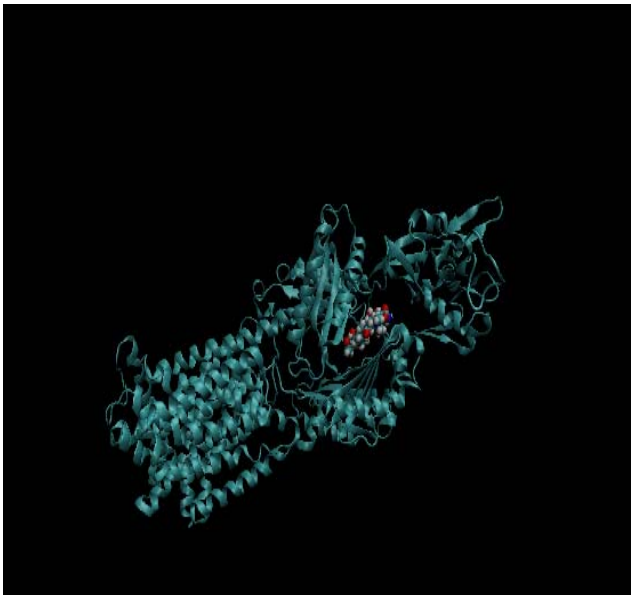

B

Figure 8 AcrB efflux pump opening and closing state upon glycosylated tetracycline binding
Shows the closing state **A.** and the opening of AcrB efflux pump **B.**, the glycosylated tetracycline is stable in the binding pocket of the target protein.

Table 1: Pharmacokinetics properties of the newly glycosylated antibiotics.

Glycosylated antibiotics	No.H bond donors	No.H bond acceptors	MW	Log p	Blood brain barrier permeability	GI absorption	Liver cytotoxicity	Lipinski's No. violations
Glycosylated Ampicillin	6	10	552.6	1.88	No	Low	No	3
Glycosylated Amoxicillin	7	11	568.6	-0.48	No	Low	No	3
Glycosylated Chloramphenicol	6	10	484.29	0.42	No	Low	No	2
Glycosylated Ciprofloxacin	5	10	492.5	2.04	No	Low	No	1
Glycosylated Tetracycline	9	14	605.59	1.34	No	Low	No	3

Table 2: Docking results of N-acetyl glucose amine antibiotics against AcrB efflux pump in *E. coli* and MexA efflux pump in *Pseudomonas aeruginosa*

Ligand	**Binding Energy (kcal/mol)**	
	ArcB	MexA
Glycosylated Amoxicillin	-8.7	-7.5
Glycosylated Ampicillin	-8.3	-7.9
Glycosylated Chloramphenicol	-8.0	-7.8
Glycosylated Ciprofloxacin	-9.0	-7.8
Glycosylated Tetracycline	-9.7	-8.8

Table 3. Binding Energy of Non- glycosylated antibiotics docked against ArcB and MexA.

Ligand	**Binding Energy (kcal/mol)**	
	ArcB	MexA
Amoxicillin	-7.8	-6.3
Ampicillin	-7.7	-6.7
Chloramphenicol	-6.1	-5.8
Ciprofloxacin	-7.5	-6.4
Tetracycline	-8.3	-6.8
PAβN	-9.3	-9.1
Pyridopyrimidine	-8.1	-9.6