RESEARCH ARTICLE

Computer-aided screening for potential inhibitory compounds against a *Klebsiella pneumoniae* local isolate containing SHV-1 and CTX-M antibiotic resistance genes

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ABSTRACT

Background: Extended-spectrum beta-lactamases (ESBLs), which allow bacteria to become resistant to commonly used antibiotics against common pathogens such as *Klebsiella pneumoniae*, are a significant public health concern as their presence severely limits treatment options. Discovery and development of new drug entities are critical to effectively combat infections with these increasingly common antibiotic-resistant variants.

Objective: Computational approaches can accelerate and reduce the cost of the discovery phase by screening for inhibitors of "druggable" pathogen enzyme targets *in silico*. In this study, protein structures of the ESBL enzymes SHV-1 and CTX-M-15 were used as targets in molecular docking experiments to identify potential inhibitors for *K. pneumoniae*.

Methodology: 5000 compounds from the Enamine Real HTS compound database were screened *in silico* for binding to SHV-1 and CTX-M-15. Twenty-six (26) compounds that were identified to have more favorable interactions compared to Avibactam, a known inhibitor of the target proteins, were tested for cytotoxic activities *in vivo* using Resazurin Microtiter Assay (REMA) against a *K. pneumoniae* clinical isolate containing both SHV-1 and CTX-M-15 resistance genes.

Results and Conclusion: Despite favorable binding energies in *in silico* screening, most of the compounds exhibited negligible inhibition on the growth of the *K. pneumoniae* clinical isolate in *in vitro* assays. This may be attributed to the fact that a phenotypic whole-cell assay, instead of an enzyme-targeted assay, was used for validation. Cell permeability requires a different set of molecular parameters which were not part of the study. Doxorubicin exhibited the highest *in vitro* bactericidal activity against this strain, which agrees with its known activity against many other bacterial pathogens and may be a promising compound for further lead optimization.

Keywords: *Antibiotic resistance, in silico drug discovery, extended-spectrum ß-lactamases*

Introduction

Every year, millions of people become infected with bacteria that are resistant to current antibiotic therapy. Second- or third-line drugs used for treating antibiotic-resistant bacterial infections are usually more expensive and may produce unwanted side effects. Infections with these antibiotic-resistant bacteria can spread uncontrollably, causing outbreaks and increasing morbidity, mortality, and health care costs of affected individuals as well as institutions [1,2]. In the Philippines, prevention of the spread of antimicrobial resistance has proven to be difficult due to several factors such as weak implementation of regulations for the proper dispensation and disposal of antimicrobials, as well as the non-compliance of healthcare providers and patients with standard antibiotic treatment guidelines [3,4].

Carbapenem-resistant *Klebsiella pneumoniae* (CR-KP) is one of the most significant emerging multidrug-resistant nosocomial pathogens as currently available β-lactam antibiotics such as penicillins, cephalosporins, and other "last line of defense" drugs do not demonstrate any *in vitro* inhibitory activity against it [5,6]. The incidence of CR-KP infections has increased dramatically worldwide since the first reported case in 1996 [5]. The resistance of CR-KP to β-lactams arises from its ability to produce β-lactamases, which are hydrolytic enzymes that disrupt the amide bond of the 4-

membered β-lactam ring found in antibiotics belonging to the penicillin and cephalosporin groups, thereby rendering them ineffective [7,8,9]. The genes coding for enzymes causing antibiotic resistance is usually located in mobile genetic elements such as transposons, integrons, and plasmids, which allows for the easy spread of resistance genes [5,6]. Resistance genes that can contribute to bacteria exhibiting extended-spectrum ß-lactamases (ESBL) properties include SHV-1 and CTX-M-15. SHV variants that belong to the ESBL phenotype are characterized by the substitution of a serine for glycine at position 238 [8]. Variants may also have a substitution of lysine for glutamate at position 240 [8,10]. SHV-1 confers resistance to broad-spectrum penicillins such as ampicillin, tigecycline, piperacillin but not the oxymino substituted cephalosporins [8,11]. CTX ß-lactamases are a family of plasmid-mediated ESBLs that preferentially hydrolyze cefotaxime [7,8].

Because of the severe public health risks posed by antibiotic-resistant strains, increasing the arsenal of antimicrobials by discovering and developing new drugs targeting ESBL-containing bacteria is of utmost priority. One of the most efficient methods in drug discovery is computer-aided drug discovery and development. Advances in genomics and proteomics have resulted in an increase in the number of potential therapeutic targets with known molecular structures that are available for investigation. Virtual, or *in silico*, screening utilizes molecular modeling to assess the interaction of compounds to a "druggable" enzyme target in the pathogen. Numerous databases for both natural products and synthetic compounds are also becoming more easily accessible. This approach can rapidly generate a host of lead compounds that can then be assayed *in vitro*.

In this study, a combination of computational and *in vitro* approaches was employed to screen for compounds that have potential bactericidal activity against a local *Klebsiella pneumoniae* isolate that contains the SHV-1 and CTX-M-15 ESBL resistance genes (unpublished data).

Methodology

In Silico Experiments

The corresponding proteins of identified genes, SHV-1 (PDB ID: 4ZAM) and CTX-M-15 (PDB ID: 4S2I), which are both in complex with the known inhibitor, Avibactam, were obtained from the Protein Data Bank (http://www.rcsb.org) [12]. The macromolecules were prepared and converted to *.pdbqt format using PyRx 0.9.6 [13]. The grid box for each protein was centered in the Avibactam binding pocket (SHV-1 grid box coordinates: X: -18.833, Y: -4.716, Z: -4.784; CTX-M-15 grid box coordinates: X: 7.401, Y: 15.918, and Z: 14.331), covering all interacting residues. Avibactam was used as the control and 5000 compounds from the Enamine Real HTS database were randomly selected using RANDOM.ORG (https://www.random.org/). The compounds were minimized and screened against the SHV-1 and CTX-M-15 protein using PyRx 0.9.6. All docking procedures were done with default parameters. Ligands with favorable binding energies in comparison to the control were subjected to ADMETox evaluation using SwissADME (http://www.swissadme.ch/) [14]. Ligands with the best ADMETox profile were selected for visual analysis. Receptor-ligand interactions were generated using Dassault Systemes Accelrys Discovery Studio Version 2017 R2. All *in silico* experiments were done using a Macintosh Desktop with OS X Yosemite Version 10.10.5, Intel® Core ™ i5 CPU 3.20 GHz processor, and 8.0 GB RAM, and a Macintosh Desktop running on an macOS Sierra Version 10.12 with an Intel® Core ™ i7 CPU 3.1 GHz processor, and 16.0 GB RAM.

Antimicrobial Assay

Commercially available compounds whose calculated binding energies were comparable to Avibactam, a known inhibitor, were purchased from Enamine, Ltd, for testing of antimicrobial activity. Antimicrobial activity was assessed using the 96-well resazurin assay format. Stock solutions of the test compounds were thawed and serially diluted using DMSO. Test compounds were assayed at high (100 ppm) and low (10 ppm) concentrations. Appropriate dilutions were placed in a well and diluted with 100 µL inoculum and 100 µL MH broth. Plates were then incubated for 24 hours at 37˚C, after which 20 µL of 0.02% v/v resazurin were added. Plates were allowed to stand for 20-30 minutes. The fluorescence of the wells was read at 520 nm excitation filter and 590 nm emission filter using FLUOstar Omega microplate reader by BMG Labtech. The extent of inhibition was computed using the equation below:

$$\%\ inhibition = 100 - 100\ [\frac{RFU_{sample} - RFU_{sterility\ control}}{RFU_{growth\ control} - RFU_{sterility\ control}}]$$

Assays were performed using four replicates per sample and their standard deviations computed. Calculations and graphs were made using GraphPad Prism Version 7.0 for Mac OS X, and data were analyzed using one-way Analysis of Variance (ANOVA) at $p < 0.05$.

Results and Discussion

In Silico Experiments

To initiate the discovery of compounds with possible inhibitory activity against ESBL-positive *Klebsiella pneumoniae*, specifically those containing SHV-1 and CTX-M, structural analysis of available protein information was first performed. It should be highlighted that for both SHV-1 and CTX-M-15, the bond between Avibactam's carbonyl carbon and N6 atom had broken due to the acylation of the serine residues of both proteins. The ligand's covalent interaction with S70, as well as its hydrogen bonds to residues S70, N170, N132, K234, T235, A237 (S237 in CTX-M-15) are conserved in both SHV-1 and CTX-M. The sulfate moiety of Avibactam displayed a strong salt bridge interaction with SHV-1 R244 [15], which is similarly produced with CTX-M-15 R276. Several other similar polar and van der Waals interactions were observed in both proteins (Table 1).

Molecular docking was done to efficiently identify potential hits that can be validated and further optimized in future experiments. Both SHV-1 and CTX-M-15 proteins were prepared, removing non-catalytic water and bound active site ligands. The ligands to be used for screening were also prepared and minimized prior to the docking procedure. Avibactam, a known β-lactamase inhibitor and the co-crystal ligand for both SHV-1 and CTX-M, was used as a control to identify the best hits from a set of 5000 compounds that were randomly selected and prepared from the Enamine HTS Database. In order to validate the docking method and generate a basis of comparison for the virtual screening procedure, the closed-ring structure of Avibactam was docked into the proteins to closely replicate X-ray crystal poses. Closed-ring Avibactam was used to simulate the complex formation as it binds to the active site with the said conformation; ring-opening and bond-breaking occur after ligand binding wherein a covalent bond forms between Avibactam and the serine residue. Grid values were set to the original binding pocket of Avibactam in the co-crystal structure and the receptor-ligand interaction diagrams of the docked complexes were analyzed. The co-crystal binding pose was suitably reproduced despite the use of the closed-ring structure of Avibactam. Moreover, while the covalent bond between the ligand and S70 of the protein binding pockets were not recreated due to the nature of the docking method used, majority of the polar and van der Waals interactions were reproduced upon docking of the ligand (Figure 1 and Table 1), indicating that the docking protocol is applicable for the protein targets and can provide viable virtual hits for the study. The predicted binding energies of the control are -7.1 kcal/mol and -6.2 kcal/mol when docked to SHV-1 and CTX-M-15, respectively.

The Enamine ligands were subjected to the same docking procedure as the control. Ligands with more negative binding energies (BE) compared to the control were identified as virtual hits (Table 2) as the stability of complexes is indicated by higher magnitudes of BE while the negative sign is indicative of the exergonic process of

Table 1. *SHV-1 and CTX-M-15 interacting water and residues with Avibactam. Residues in blue are present in both the co-crystallized open ring form and the closed-ring docked form.*

Protein	Avibactam Structure	Interacting Water and Residues
SHV-1	Open-ring form (co-crystallized)	M69, S70, S70 (covalent), K73, Y105, S130, N132, E166, T167, N170, V216, T235, G236, K234, A237, R244, HOH420, HOH494, HOH528, HOH525, HOH543, HOH546, HOH601, HOH629
	Closed-ring form (docked)	S70, Y105, S130, N132, E166, T167, N170, V216, T235, G236, A237, G238, M272, HOH420, HOH601
CTX-M-15	Open-ring form (co-crystallized)	C69, S70, S70 (covalent), K73, N104, Y105, S130, N132, N170, T216, K234, T235, G236, S237, G238, R276, HOH401, HOH412, HOH425, HOH492, HOH516, HOH551
	Closed-ring form (docked)	S70, K73, N104, Y105, S130, N132, N170, T216, K234, T235, G236, S237, G238, HOH401, HOH412, HOH425, HOH492, HOH515, HOH516, HOH647

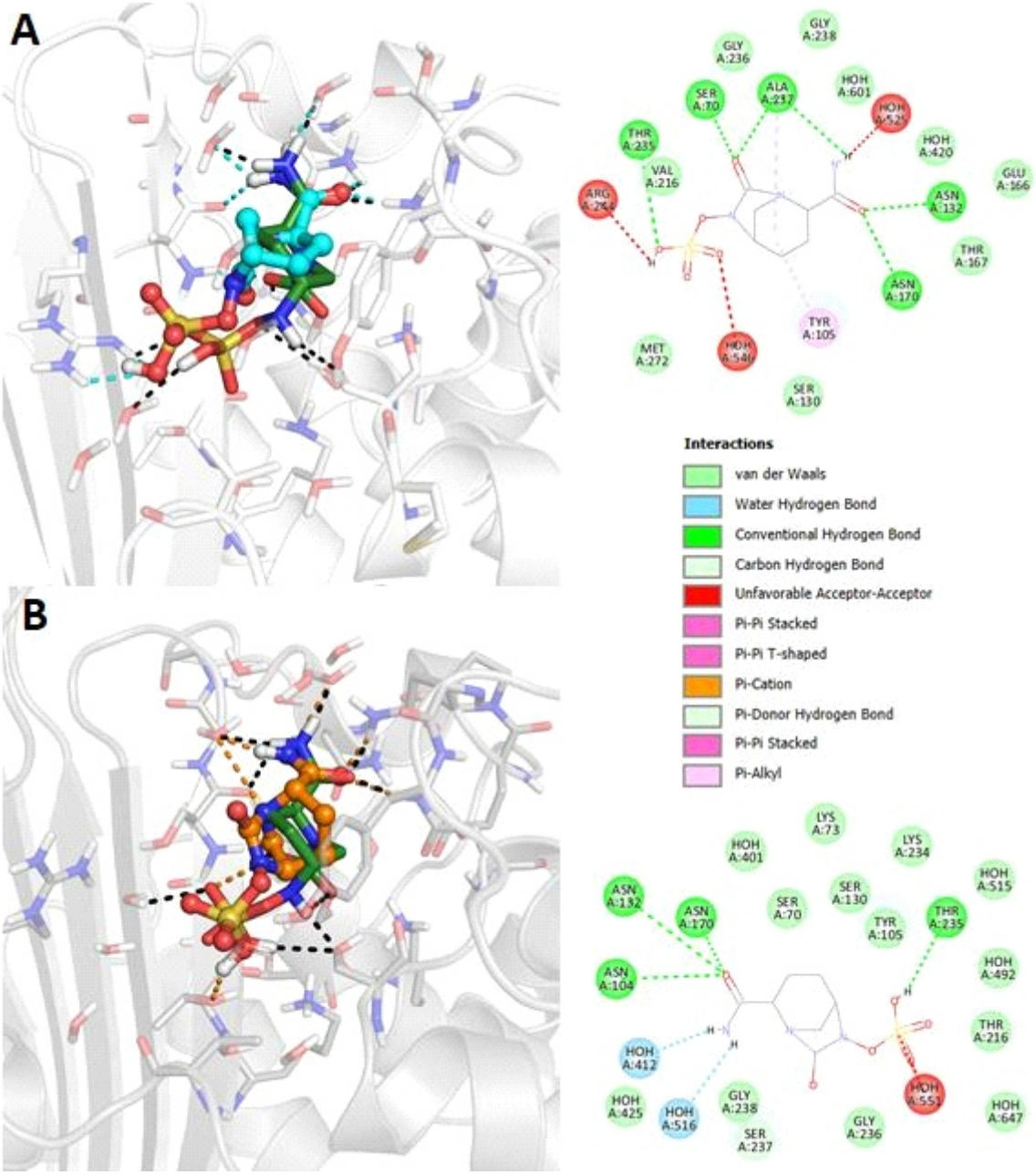

Figure 1. *Overlay of docked Avibactam with co-crystallized Avibactam in (A) SHV-1 and (B) CTX-M-15 with corresponding 2D ligand interaction diagrams. Co-crystal Avibactam is displayed as sticks with green carbon atoms. Avibactam complexed with SHV-1 and CTX-M-15 are shown in ball-and-sticks representation with cyan and orange carbon atoms, respectively. Binding pocket polar interactions are shown as black (co-crystal), cyan (SHV-1-bound), and orange (CTX-M-15-bound) dashed lines. 2D ligand diagrams show interactions between the ligand and residues and waters found in the binding pocket. Light purple halos and circles indicate solvent accessibility while the rest of the legend is included in the figure.*

complex formation [16]. Z24206142 exhibited the best binding energies from the docking experiments, with -8.4 kcal/mol and -8.8 kcal/mol for SHV-1 and CTX-M, respectively. As seen in Figure 2, the chromenone group of Z24206142 notably displayed pi-pi stacking interactions with Y105 in both SHV-1 and CTX-M-15. The carbonyl of the chromenone group acted as a hydrogen bond acceptor for a water molecule found in the SHV-1 binding site. The ester connecting group of the ligand formed hydrogen bonds with S70 and A237, which are some of the key interactions of Avibactam with SHV-1. The absence of a charged group in Z24206142 in comparison to the control did not preclude the formation of a strong interaction with SHV-1 R244, which displayed a pi-cation interaction with the isoindoline moiety.

Table 2. *Binding energies of compounds considered as top hits based on binding energies calculated from molecular docking interactions between each compound and target protein (SHV-1 and CTX-M-15).*

Index	Ligand	Binding Energy (kcal/mol)	
		SHV-1	CTX-M-15
Control	Avibactam	-7.1	-6.2
1	Z24206142	-8.4	-9.1
2	Z114097922	-7.7	-9.1
3	Z114950210	-7.7	-8.5
4	Z1172205719	-8.0	-8.4
5	Z152078990	-7.9	-8.4
6	Z19178756	-7.8	-8.4
7	Z116334870	-7.8	-8.3
8	Z152043348	-7.7	-8.1
9	Z1607324591	-7.7	-8.1
10	Z19288438	-8.8	-8.0
11	Z1172318344	-7.8	-8.0
12	Z1312720210	-7.9	-8.0
13	Z2061563625	-7.7	-8.0
14	Z2704932606	-7.9	-8.0
15	Z126251738	-8.1	-7.9
16	Z151946554	-8.0	-7.9
17	Z152080570	-7.7	-7.9
18	Z16957737	-7.7	-7.8
19	Z152015262	-7.7	-7.8
20	Z152015608	-7.7	-7.8
21	Z227665298	-7.7	-7.8
22	Z1212697754	-7.9	-7.7
23	Z152043418	-7.8	-7.7
24	Z1568904421	-7.8	-7.7
25	Z238075486	-7.7	-7.7
26	Doxorubicin	-8.0	-7.9

Pi-alkyl interactions are also observed between the ligand isoindoline group and M272 and A237, while the rest of the interactions found in the binding pocket are van der Waals contacts. In the CTX-M-15 binding site, the Z24206142 chromenone group formed an H-bond with N104 and N132, while the carbonyl oxygen from the connecting ester formed an H-bond acceptor interaction with K234. The difference in binding orientation compared to binding with SHV-1 led to a weaker van der Waals interaction with R276. While the ligand displayed an unfavorable interaction with T235 and had less extensive electrostatic interactions within the CTX-M-15 pocket, it displayed more van der Waals interactions with the residues and water molecules within the binding site than when it was SHV-1-bound. These van der Waals interactions, in spite of their lower individual energy contribution compared to H-bond or pi interactions, can effectively accumulate to account for the better binding energy with CTX-M-15. These results collectively suggest that Z24206142 is a viable hit compound that can be developed to target ESBL-positive *Klebsiella pneumoniae*.

In silico ADME prediction using the SwissADME server (Table 3) showed that Z24206142 has good GI absorption, solubility, and synthetic accessibility. It was also found to be drug-like with low target promiscuity. However, it was predicted to inhibit a number of CYP450 enzymes including CYP1A2, CYP2C19, CYP2C9, and CYP3A4 suggesting that it may interfere with the metabolism of a number of commonly used drugs. Regardless, its low calculated lipophilicity and drug-likeness indicate that there is still a lot of room for structural optimization to improve its pharmacokinetic properties.

Antimicrobial Assay

The cytotoxic activities of shortlisted compounds from Enamine and of doxorubicin were determined using resazurin microtiter assay (REMA) against a *Klebsiella pneumoniae* clinical isolate obtained from St. Luke's Medical Center which contains both SHV-1 and CTX-M-15 ESBL genes (unpublished data). Among all the compounds tested, doxorubicin had the best activity, producing 52.52% inhibition at 100 ppm. This was followed by Z238075486 which showed inhibitory activity at 27.96% at the same concentration (Figure 3).

Despite favorable binding energies measured in the *in silico* screening, most of the compounds exhibited negligible

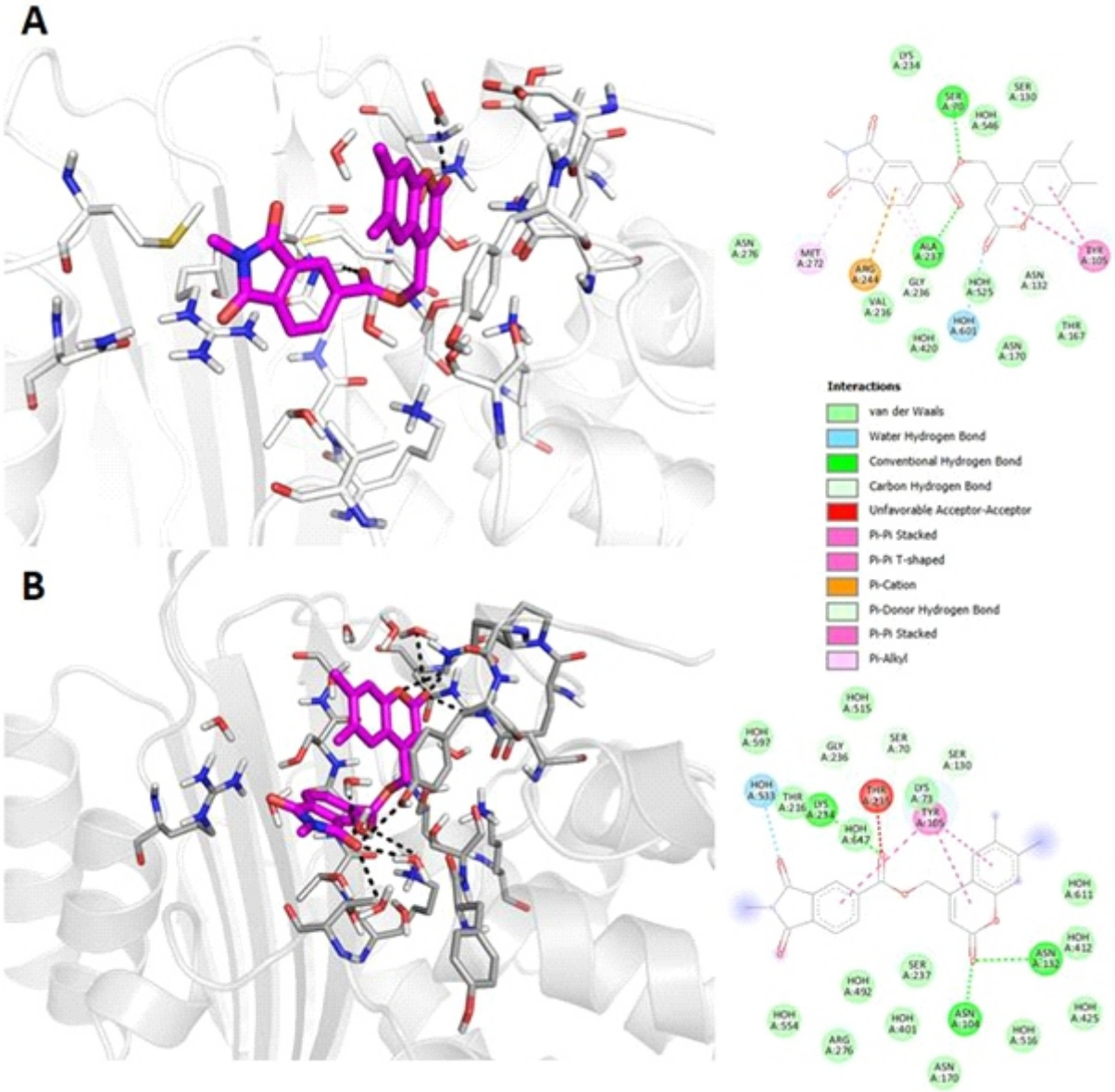

Figure 2. *Z24206142 in complex with (A) SHV-1 and (B) CTX-M-15 with corresponding 2D ligand interaction diagrams. Z24206142 is displayed as sticks with magenta carbon atoms. Binding pocket polar interactions are shown as black dashed lines. 2D ligand diagrams show interactions between the ligand and residues and waters found in the binding pocket. Light purple halos and circles indicate solvent accessibility while the rest of the legend is included in the figure.*

inhibition on the growth of *Klebsiella pneumoniae* in the *in vitro* assay. This may be attributed to the fact that a phenotypic whole cell assay, instead of an enzyme assay that directly assesses interaction with the potential inhibitory compound, was used for validation. For the potential inhibitor to reach the target enzyme, it will have to enter the bacterial cell first. Cell permeability requires a different set of molecular parameters which were not taken into account in this study. Moreover, there are numerous bacterial virulence factors which the compounds may have been unable to overcome. The main virulence factor that could have contributed to the ineffective activity of the compounds on *Klebsiella pneumoniae* is the presence of capsular serotypes and efflux pumps [17,18]. Other possible factors are lipopolysaccharides, siderophores, and fimbriae. The capsular polysaccharide imparts increased virulence to the bacteria by providing a layer that impairs the activity of bactericidal action of serum and provides a defense against phagocytosis, which is a major part of the body's secondary line of defense [17]. Over 70 types of capsular serotypes have been reported for *Klebsiella pneumoniae*. In particular, K1 and K2 serotypes, which mostly cause liver abscess, are hypermucoviscous (HV).

Table 3. *In silico ADME properties of Z24206142 predicted using the SwissADME server.*

Property	Predicted value
iLOGP	3.11
Log S (ESOL)	3.99 x 10-2 mg/mL (soluble)
GI absorption	High
BBB permeant	No
P-gp substrate	No
CYP1A2 inhibitor	Yes
CYP2C19 inhibitor	Yes
CYP2C9 inhibitor	Yes
CYP2D6 inhibitor	No
CYP3A4 inhibitor	Yes
Lipinski	0 violation (druglike)
PAINS	0 alert
Synthetic accessibility	3.28

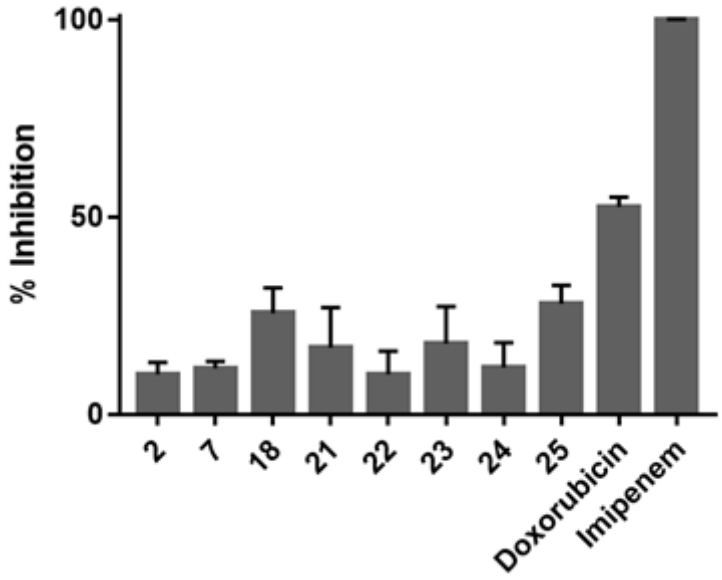

Figure 2. *Percent Inhibition of nine shortlisted compounds assayed at 100 ppm, as measured in the resazurin assay using a 96-well plate format. Compounds that have a % inhibition of less than 10% were not shown. Enamine code names for the compounds are listed in Table 3. Imipenem was used as the positive control. Values are shown as mean ± SD (n=4).*

The intrinsic resistance of gram-negative bacteria to antibiotics has also been attributed to efflux pumps which are transport proteins that shuttle toxicants [19]. In the presence of antibiotics, it is possible that efflux pumps become overexpressed to ensure the transport of any antibiotics or toxicants out of the cell. The synergistic activities of capsular serotypes, efflux pumps, resistance genes, and the mutation of target sites for antibiotics can lead to highly resistant bacteria that may be even more difficult to treat.

Conclusion

In this study, the enzymes expressed by SHV-1 and CTX-M-15 were used as druggable targets for molecular docking experiments to identify potential inhibitory compounds that can trigger bacterial cell death. Out of 5000 compounds, 26 ligands were found to have favorable binding activity and were shortlisted for cytotoxicity assays. Of these, only doxorubicin exhibited percent inhibition above 50% at 100ppm against a local clinical isolate found to contain both SHV-1 and CTX-M-15 resistance genes. Low cell permeability and the bacteria's virulence factors (e.g., capsular serotypes and efflux pumps) may have prevented sufficient inhibitory activity on the target enzymes. Capsular serotypes impart increased virulence by impairing the activity of bactericides and providing a defense against phagocytosis. In order to more fully assess enzymatic activity of compounds found to have good binding activity to the target proteins, future

studies may include targeted *in vitro* enzymatic binding assays. Computational studies that can determine the ability of a compound to pass through bacterial cell walls and membranes may also be employed prior to *in vitro* assays. Future studies may also be done in order to determine synergistic effects, if any, with commonly used inhibitors like clavulanic acid, cilastatin, as well as other currently used antibiotics such as ampicillin and imipenem.

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