International Research Journal of Biochemistry and Bioinformatics Vol. 1(4) pp. 095-102, May, 2011
Available online http://www.interesjournals.org/IRJBB

Full Length Research Paper

Molecular modeling and virtual screening studies of NDM-1 Beta lactamase for identification of a series of potent inhibitors.

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Accepted 4 May, 2011

NDM-1 metallo-β-lactamase (class B) is a plasmid-borne zinc metalloenzyme that efficiently hydrolyzes β-lactam antibiotics, including carbapenems, rendering them ineffective. Resistance to β -lactam antibiotics mediated by metallo-β-lactamases is an increasingly worrying clinical problem. Because NDM-1 has been found in several clinically important carbapenem-resistant pathogens, there is a need for inhibitors of this enzyme that could protect broad spectrum antibiotics from hydrolysis and thus extend their utility. In the presented research, the 3D structure of NDM-1 protein was modeled using homology modeling by Modeller9v5. Evaluation of the constructed NDM-1 protein model was done by PROCHECK, WHATCHECK, ERRAT, VERIYFY3D and through ProSA calculations. A compound library screening approach was used to identify compounds from the ZINC Database and characterize NDM-1 inhibitors from a library of active compounds. The strategy employed was divided into two categories, viz. screening and docking. A series of compounds from ZINC Database have been identified as potent inhibitors of NDM-1 metallo-β-lactamase.

Keywords: NDM-1 metallo-β-lactamase, homology modeling, ZINC Database, virtual-screening, docking, inhibitor.

INTRODUCTION

The β-lactamase isolate from New Dehli has been named NDM-1 or blaNDM-1, which stands for New Delhi Metallo-β-lactamase. The multi-drug resistant *Klebsiella pneumoniae* containing NDM-1 has thus been named a 'superbug'. NDM-1 is mainly found in *K. pneumoniae*, but recently other bacteria such as *Enterobacteriaceae* and *Acinetobacter baumannii* display NDM-1 activity as a result of the genetic plasticity of the plasmid that carries the genes responsible for producing the resistant mechanisms (Yong et al., 2009). NDM-1 metallo-β-lactamase provides bacteria with an efficient and effective way of mediating resistance to β-lactam-based antibacterial agents. More significantly, they confer resistance to carbapenems. Therefore, if metallo-β-lactamases increase in prevalence, they could compromise the efficacy of this group of antibiotics to treat life-threatening hospital infections. β-lactams have been the mainstay of treatment for serious infections, and the most active of these are the carbapenems, which are advocated for use for the treatment of infections caused by ESBL producing *Enterobacteriaceae*, particularly *Escherichia coli* and *Klebsiella pneumonia* (Paterson, 2006). Metallo-β-lactamases require zinc ions to catalyse the hydrolysis of β-lactams (Zn^{2+}-dependent β-lactamases). The active site of MBLs is situated at the bottom of a wide shallow groove between two β-sheets and has two potential zinc-ion binding sites at the active site. MBLs are divided into three classes based on their Zn^{2+} dependency, i.e., whether they (i) are fully active with either one or two ions (subclass B1, e.g., IMP-1, VIM-2, BcII, CcrA and NDM-1), (ii) require two ions (subclass B3, e.g., L1), or (iii) employ one ion and are inhibited by binding of an additional ion (subclass B2, e.g., CphA) (Drawz et al. 2010).

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The active site for VIM-2 enzyme class is formed by a shallow cleft containing one or two Zn^{2+} cofactor ions and two flexible loops. The first loop (Phe61 to Ala64) makes key aromatic and hydrophobic interactions with the bound inhibitor through residues Phe61 and Tyr67, while the other loop (Ile223-Trp242) undergoes a ligand-induced side chain reorientation of Asn233 (Minond et al., 2009). NDM-1 shares little identity with other MBLs, with the most similar MBLs being VIM-1, VIM-2, VIM-4, SIM-1 and IMP-1. NDM-1 not only is a new subclass of the B1 group of MBLs but also possesses novel amino acids near the active site, suggesting that it has a novel structure. NDM-1 possesses only 32.4% identity with VIM-2, NDM-1 also has a unique HXHXD motif among the mobile MBLs, as it contains an alanine between the two histidines (Yong et al., 2009).

There are currently no clinically useful inhibitors of NDM-1 metallo-β-lactamase; however, for metallo-β-lactamases several studies have been undertaken with a variety of experimental inhibitors. These can be divided into (i) compounds which irreversibly covalently modify the enzyme, resulting in inhibition or inactivation of activity, (ii) compounds which chelate zinc from the active site, resulting in reversible inactivation of the enzymes, or (iii) compounds which competitively inhibit substrate binding, either by mimicking the structure of the β-lactam substrate or by co-ordinating to an active-site zinc ion, preventing binding of the β-lactam substrate or displacing a bound substrate (Simm et al., 2005). Inhibitors that covalently modify metallo-β-lactamases include small thiol-modifying reagents, such as mercuric (II) salts (Bush et al., 1995), p-chloromercuribenzoate (Bush, 1989), iodoacetic acid (Payne, 1993) and mercaptoacetic acid thiol esters (Payne et al., 1997). Inhibitors that chelate the active-site zinc include EDTA, 1, 10-phenanthroline, dipicolinic acid, two phenazines from Streptomyces spp. (Gilpin et al., 1995), bis(1Ntetrazol- 5-yl)amine (Toney et al., 1998) and EGTA (Laraki et al., 1999). More promising compounds are those which reversibly block the active site by competitive inhibition, since these offers the potential for modification of the structure to improve the specificity of the inhibitor for metallo-*β*-lactamases alone. Such compounds include biphenyl tetrazoles (Toney et al., 1998), mercaptophenyl acetic acid derivatives (Payne, 1993), thiomandelic acid (Mollard et al., 2001) and thioxo-cephalosporin derivatives (Tsang et al., 2004).

Till date, crystal structure of NDM-1 metallo-β-lactamase is not present in public repository databases, so determining the 3D structure provides a new opportunity for the discovery of more potent inhibitors, particularly in the application of structure based virtual screening to identify lead compounds. To this end, an approach has been taken that combines identification of 3D structure of NDM-1 protein and computational docking process to identify a series of potent inhibitors from ZINC Database.

METHODOLOGY

Sequence alignment

The protein sequence of NDM-1 (UniProt ID: C7C422) was obtained from Swiss Prot database (http://www.uniprot.org/uniprot) and protein sequences of IMP-1 (GenBank ID: ABG67754), VIM-1 (GenBank ID: CAG23926), VIM-2 (GenBank ID: ACH43053) and VIM-4 (GenBank ID: ABC97285) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Multiple sequence alignment of the amino acid sequences of NDM-1 metallo-β-lactamase with the amino acid sequences of IMP-1, VIM-1, VIM-2 and VIM-4 were performed with the online version of CLUSTALW (Larkin et al., 2007) 1.81 program to identify the set of conserved residues in the alignment (Figure 1).

Template identification and protein homology modeling

A search of the RCSB Protein Data Bank (http://www.rcsb.org/pdb) confirmed that neither the X-ray crystal structure of NDM-1 metallo-β-lactamase, nor the co-crystallized structure with an inhibitor is publically available from *K.pneumoniae*. Primarily, the search began with finding of a number of related sequences by the BLASTP program to reveal NDM-1 related three dimensional structures as a template (Altschul et al., 1997). The most suitable template was selected for the study. High-resolution X-ray crystallographic structure of Di-Zinc metallo-β-lactamase Vim- 4 from *Pseudomonas Aeruginosa* (PDB ID: 2WHG) was the selected template protein for homology modeling. Modeller9v5 (Šali et al., 1993) was used to construct the 3D models of NDM-1 protein. Final homology model was selected on the basis of MOLPDF, DOPE score GA341 score.

Model optimization and evaluation

The protein models generated by homology modeling often produce unfavorable bond lengths, bond angles, torsion angles and contacts. Therefore, it was essential to minimize the energy to regularize local bond and angle geometry as well as to relax close contacts in geometric chain. Each model of NDM-1was optimized with the variable target function method (VTFM) with conjugate gradients (CG) followed by further refinement by using the molecular dynamics (MD) with simulated annealing (SA) method in Modeller itself (Šali et al., 1993). The energy minimization was performed to relieve stearic collisions and strains without significant alterations in the overall structure. Energy computations and minimization were done with the GROMOS96 (Scott et al., 1999) force

Figure 1. Alignment of the amino acid sequences of NDM-1 with the amino acid sequences of VIM-1, VIM-4, VIM-2 and IMP-1. Conserved residues coordinating with zinc ions are denoted with asterisks enclosed in yellow box. The results were generated with Clustal W multiple sequence alignment tool.

field by implementation of Swiss-PdbViewer (http://www.expasy.org/spdbv). After the optimization procedure, the 3D model of NDM-1 was verified by using PROCHECK (Laskowski et al., 1993), ERRAT (Colovos et al., 1993) and VERIFY 3D (Bowie et al., 1991) (Lüthy et al., 1992) programs of Structural Analysis and Verification Server (SAVES) (http://nihserver.mbi.ucla.edu/SAVES). The PROCHECK assessed the "stereo chemical quality" of the protein structure. The Verify3D program assessed the 3D protein structure using three-dimensional profiles by analyzing the compatibility of an atomic model (3D) with its own amino acid sequence (1D). The quality of model was also validated by ProSA (Wiederstein et al., 2007) (Sippl, 1993) server (https://prosa.services.came.sbg.ac.at/prosa.php), a web server for Protein Structure Analysis.

Active site analysis

Usually, active site structure is highly conserved among distantly related enzymes. The catalytic activity of an enzyme is performed by a small, highly conserved constellation of residues within the active site. The β-lactamases in the class B require one or two zinc ions for their full catalytic activity (Walsh et al., 2005) (Crowder et al., 2006). Active site identification included the superimposition of the model with template which provided integrity of the homology model and assisted in positioning conserved active site residues. The model was overlapped with the template to obtain active site's information for the modeled structure and conserved active site residues were also verified manually in multiple-aligned-sequences of other members of β-lactamases (Figure 1).

Screening of compounds from ZINC Database

Virtual screening techniques continue to play an important role in the lead discovery process. The key point of this technology encompasses the ability to screen compound databases in the active sites of protein with a 3D structure. The ZINC Database (http://zinc.docking.org/) is a curated collection of commercially available chemical compounds prepared especially for virtual screening. It contains over 13 million

compounds. During our work ZINC Database was used for screening of structurally similar inhibitors of class B, or metallo--β--lactamases. These compounds included (i) N-arylsulphonyl hydrazones (Siemann et al., 2002), (ii) Thiomandelic acid (Mollard et al., 2001), (iii) Mercaptocarboxylate inhibitors (Thiol Derivatives), (iv) Thioxocephalosporin and Penicillin-derived inhibitors, (v) Biphenyl Tetrazole inhibitors, (vi) Trifluoromethyl Ketones and Alcohols, (vii) Carbapenem Analogs, (viii) Succinate Derivatives, (ix) Tricyclic natural products, (x) C-6-Mercaptomethyl Penicillinates (Drawz et al. 2010), (xi) Mercaptophenyl acetic acid derivatives (Tsang et al., 2004), and (xii) Sulfonyl-triazole compounds (Minond et al., 2009). After screening, a total of 1295 compounds were obtained that were structurally similar to available inhibitors of metallo-β-lactamases. The screening of database was performed by providing molecular constraints (property based search) and the physico-chemical properties such as log P value, H-bond donors, H-bond acceptors, molecular weight and rotational bonds (Lipinski's rule) were also kept into consideration.

Structure based virtual screening using molecular docking

Virtual screening was performed by docking of inhibitors obtained from ZINC Database to the active site of NDM-1 protein by using AutoDock Vina software (Trott et al., 2010). Docking was performed to obtain a population of possible conformations and orientations for the ligand at the binding site. Using the Autodock Tools (Goodsell et al., 1990) (http://autodock.scripps.edu/resources/adt) software, polar hydrogen atoms were added to the NDM-1 protein and its nonpolar hydrogen atoms were merged. The protein receptor (NDM-1) and the inhibitors were converted from PDB format to PDBQT format. All bonds of ligands were set to be rotatable except N-C bonds. In the configuration file of Autodock Vina software, the grid box with a dimension of 20 x 20 x 20 points was used around the active site to cover the entire enzyme binding site and accommodate ligands to move freely. The best conformation was chosen with the lowest docked energy, after the docking search was completed. The interactions of complex NDM-1 protein-ligand conformations, including hydrogen bonds and the bond lengths were analyzed using Swiss-PdbViewer (Guex et al., 1997) v4.0, Pymol software (http://www.pymol.org), UCSF Chimera (http://www.cgl.ucsf.edu/chimera) and Accelrys DS Visualizer software (http://accelrys.com).

RESULTS

Protein homology modeling

Homology search for NDM-1 metallo-β-lactamase of *K. pneumoniae* resulted into a large number of sequences by running BLASTP against PDB database. The target sequence showed an identity of 38% with metallo-β-lactamase VIM-4 from *P. Aeruginosa* (PDB ID: 2WHG). Chain A of the template was used as a template for making 3D models of NDM-1 protein in Modeller. The sequence similarity of *K.pneumoniae* NDM-1 metallo-β-lactamase with *P.Aeruginosa* VIM-4 strongly advocates that these two enzymes belong to a common class B1 of metallo-β-lactamases. From a total of five 3D-models of NDM-1 metallo-β-lactamase, the best 3D-model (Figure 2) had Molpdf value of 1077.94666, DOPE score of -23736.64258 and GA341 score of 1.00000. The quality of the 3D model was evaluated using the PROCHECK program and assessed using the Ramachandran plot (Figure 3). It is evident from the Ramachandran plot that the predicted model has most favorable regions, the allowed regions, the generic regions and the disallowed regions. Such a percentage distribution of the protein residues determined by Ramachandran plot shows that the predicted model is of good quality. The model show all the main chain and side chain parameters to be in the 'better' region. The overall quality factor of 3D model predicted by ERRAT server was 85.167. Verify 3D server predicted that 95.45% of the residues in NDM-1 metallo-β-lactamase had an averaged 3D-1D, so the model was also verified by the Verify 3D. The quality of the 3D model of NDM-1 metallo-Beta-lactamase as evaluated by ProSA web server (https://prosa.services.came.sbg.ac.at/prosa.php) provided a z-score of -7.77 which falls within the range of values observed for the experimentally determined structures of similar lengths.

Active site analysis

The information of active site was obtained by superimposing 3D models of the target protein with template protein, which provided reliability of homology between the 3D structures. The super positioning of protein 3D models also aided in depicting the conserved active site residues among the query and the template. Active site information of template structure was obtained from the literature (Galleni et al., 2001) and overlapped with target protein (Figure 4). The amino acid residues H120, H122, H189 and D124, C208, H250 make the first and second zinc binding motif respectively. The information was obtained on the basis of multiple sequence alignment of the respective members of class B1 Beta lactamases. Thus, active site forming residues were found to be highly conserved among B class of Beta lactamases.

Virtual Screening result analysis

The most important requirement for interaction of NDM-1 protein and inhibitors was the proper orientation and

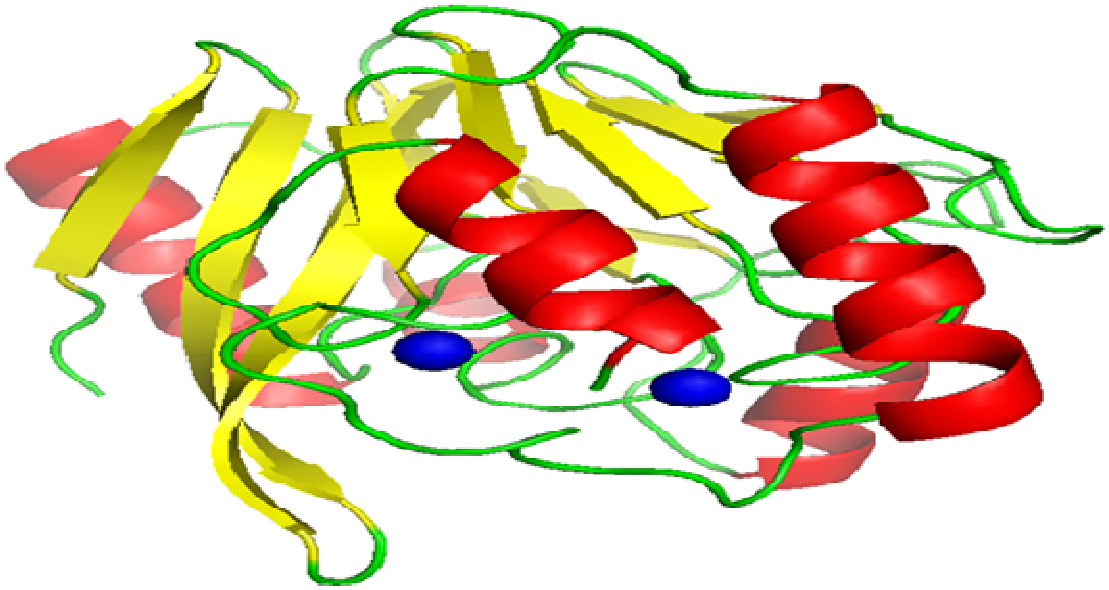

Figure 2. The best 3-D model of NDM-1 metallo-β-lactamase generated by modeler represented by Pymol software in cartoon view (Red: Helices, Yellow: Strands, Green: Loops, Blue spheres: Zinc ions).

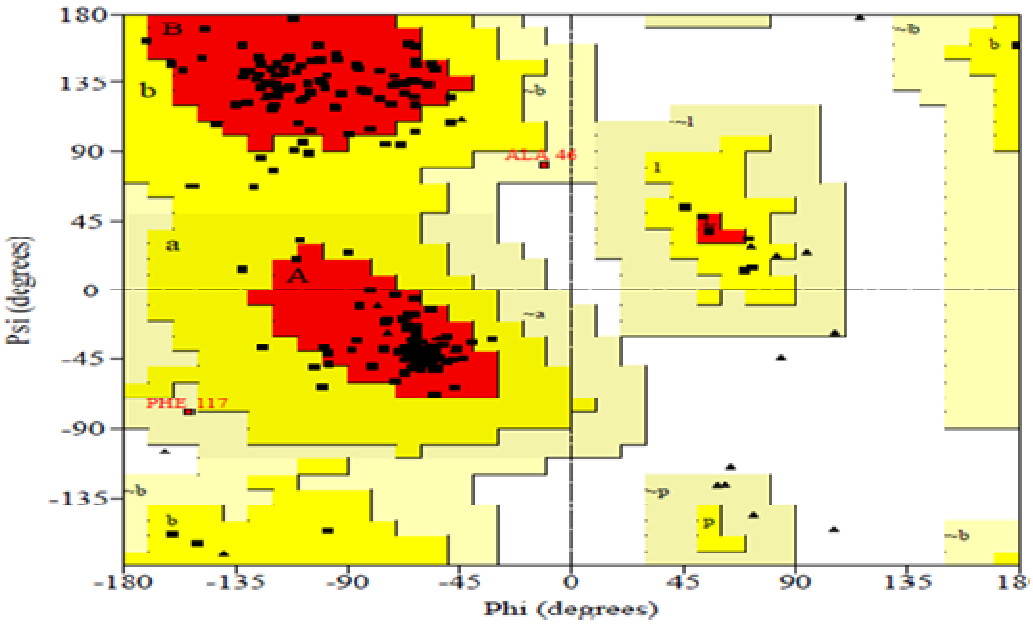

Figure 3. Ramachandran plot of NDM-1 metallo-β-lactamase 3D model obtained by PROCHECK validation package.

conformation of inhibitor into the NDM-1 enzyme active site. A total of 1295 compounds were obtained from ZINC Database in mol2 format. The best 6 inhibitors (Figure 5) were selected on the basis of binding affinity and the extent of binding towards NDM-1. The selected inhibitors from ZINC Database were structurally similar to N-

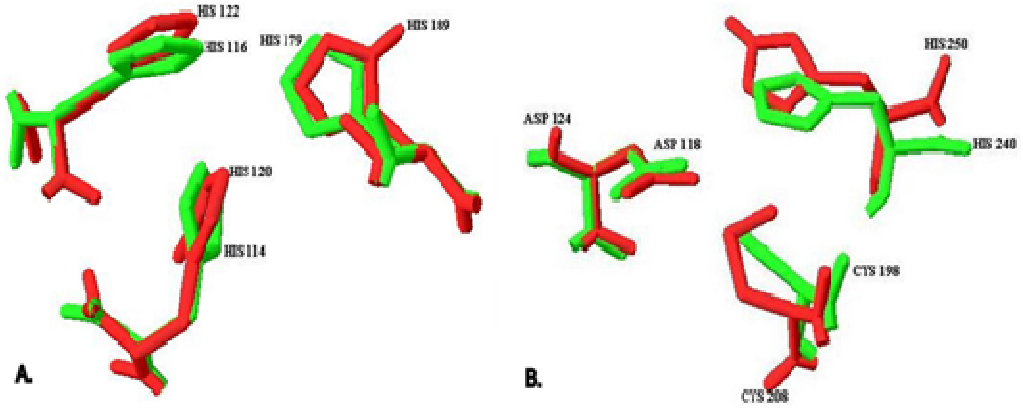

Figure 4. Superimposition of active site residues of template (2WHG-A) and modeled protein NDM-1. Green and red color sticks represents template and modeled proteins respectively. Figure 4(A) and 4(B) represents the first and second Zinc binding motif respectively.

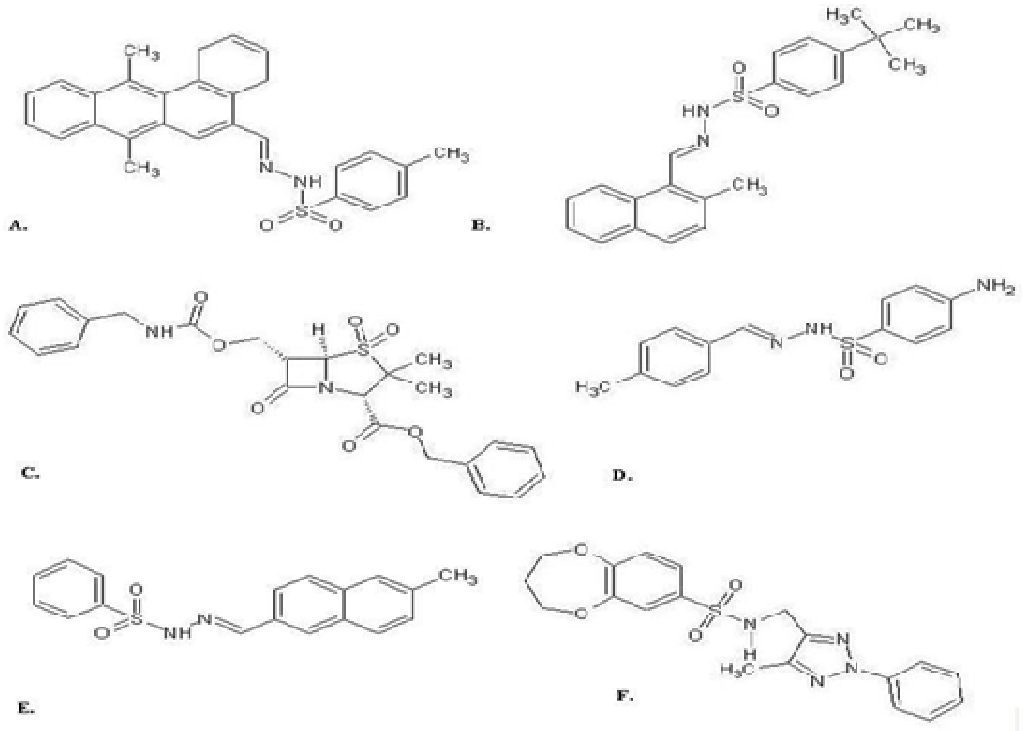

Figure 5. Structures of inhibitor compounds from ZINC Database that showed maximum binding affinity for NDM-1 metallo-β-lactamase (5(A): ZINC8628455, 5(B): ZINC1155856, 5(C): ZINC26384998, 5(D): ZINC1768686, 5(E): ZINC83977, 5(F): ZINC 1034904).

arylsulphonyl hydrazones, Sulfonyl-triazole and C-6-Mercaptomethyl Penicillinates among 12 categories of metallo--β—lactamase inhibitors taken into consideration. The optimal interactions and the best affinity score were used as criteria to interpret the best conformation among the 10 generated conformations for each inhibitor from ZINC Database. Overall, the best confirmation showed that the free energy of binding (ΔGbind kacl/mol) for the

Table1. The binding affinity and the number of bonds formed in the protein- inhibitor complexes.

ZINC ID	Binding Affinity (Kcal/mol)	Bond(s) with Zn atom
ZINC 8628455	-9.0	2 pi bonds
ZINC 1155856	-8.9	1 H bond
ZINC 26384998	-8.8	1 pi bond
ZINC 1768686	-8.8	1 pi bond
ZINC 83977	-8.7	1 pi bond
ZINC 1034904	-8.6	1 pi bond, 1 H bond

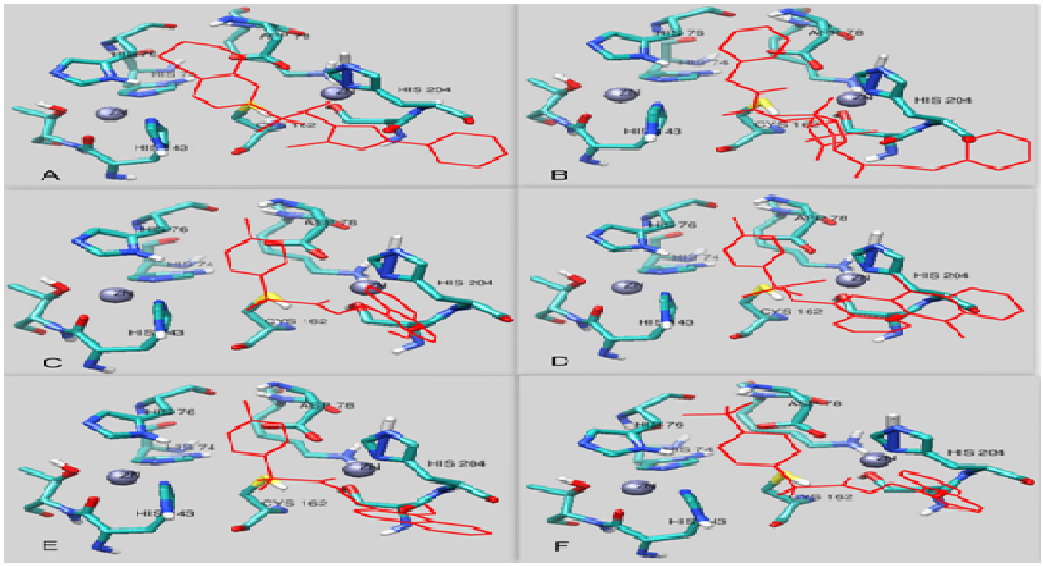

Figure 6. Proposed mode of binding of inhibitor compounds to NDM-1 protein represented with Chimera software. The active sites of NDM-1 are depicted in sticks model and the two zinc ions are represented as purple spheres. The inhibitors (6(A): ZINC 1034904, 6(B): ZINC26384998, 6(C): ZINC1768686, 6(D):ZINC8628455, 6(E): ZINC83977, 6(F): ZINC1155856) are shown in red color.

best 6 inhibitors were good and represented in Table 1 as compared to N-arylsulphonyl hydrazones (-5.8 kcal/mol), Sulfonyl-triazole (-7.8 kcal/mol) and C-6-Mercaptomethyl Penicillinates (-6.3 kcal/mol). The negative and low value of ΔGbind indicated strong favorable bonds between NDM-1 and the inhibitors and indicated that the inhibitors were in their most favourable conformations. The analysis of the docked complexes showed that the inhibitors were located near the active site and were stabilized by hydrogen bonds and pi bonds with zinc ions. Figure 6 represents the docked conformation of the selected ZINC Database inhibitor compounds with NDM-1protein.

DISCUSSION AND CONCLUSION

NDM-1 metallo-β-lactamase is shown to be most potent cause for antimicrobial resistance in bacteria. Virtual screening methods are routinely and extensively used to reduced cost and time of drug discovery process. It has been clearly demonstrated that the approach utilized in this study is successful in finding 6 potent inhibitors from the ZINC Database. In this work, 3D model of NDM-1 metallo-β–lactamase was predicted and it was used for screening potent compounds from ZINC Database. Docking results indicate that out of 1295 compounds, there were 6 inhibitory compounds for NDM-1 metallo-β–lactamase that showed interaction with the protein to a great extent and these inhibitors could be used against NDM-1 metallo-β-lactamase. Hydrogen bonding plays an important role for the structure and function of biological molecules, especially for inhibition in a complex. The inhibitors were docked deeply within the binding pocket region and forming interactions. Therefore, this study states that the N-arylsulphonyl hydrazones, Sulfonyl-triazole and C-6-Mercaptomethyl Penicillinates derivatives have the potency to inhibit the NDM-1 protein and the role of small molecule libraries to enhance drug discovery process. Further, this work can be extended to

study the receptor inhibitor interactions experimentally and evaluation of their biological activity would help in designing compounds based on virtual screening techniques.

ACKNOWLEDGMENT

The authors would like to acknowledge the support provided by Mr. Vikas Rana for his help to improve the quality of pictures in this manuscript.

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