Tropical Biomedicine 33(1): 180–189 (2016)

Antibiotics as Japanese Encephalitis virus inhibitors: a combinatorial computational approach

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Received 12 December 2014; received in revised form 30 December 2015; accepted 10 January 2016

Abstract. Japanese encephalitis (JE) is a viral neurologic disease of global public health importance. JE is caused by an RNA virus – the Japanese encephalitis virus (JEV) belonging to genus *Flavivirus* in the family *Flaviviridae*. JE is endemic in many parts of Asia and western pacific. Annually, approximately 50,000 JE cases are reported with case fatality rates as high as 30-35% resulting in ~15,000 deaths. Presently, there are no successful drugs against JE. Docking of JEV NS3 helicase/NTPase helicase domain with 10 compounds was performed in iGEMDOCK v2.1. Integrated docking, screening, post- analysis and visualization were performed using RasMol software. The drug susceptibility was evaluated by virus yield reduction assay. Three ligands out of 10 antibiotic compounds studied showed highest binding affinity with receptor protein. Kanamycin, Rolitetracycline and Doxycycline showed better binding energy compared to two study standards- Ribavirin and Minocycline. Interacting bonds were formed in all three domains of NS3 helicase/NTPase. The interactions in motifs I, II and VI of helicase are important; these would have possibly inhibited viral replication. Biological assay showed that Kanamycin (Inhibitory concentration$_{50}$, IC_{50} – 70 µg/ml), Rolitetracycline (IC_{50} – 76 µg/ml) and Doxycycline (IC_{50} – 22 µg/ml) inhibited plaque formation.

INTRODUCTION

Japanese encephalitis (JE) is an arthropod borne viral disease of global public health importance. The disease is prevalent in Southeast Asia and the Pacific (Solomon, 2006). However, it is feared that JE could become a global pathogen and causing worldwide pandemics (Ghosh & Basu, 2009). JE affects the central nervous system, at times leading to irreversible neurological damage in humans (Erangler *et al.*, 2009). Annually, an estimated 50,000 cases with ~ 15,000 deaths are recorded (Tsai, 1997). JE is caused by Japanese encephalitis virus (JEV) which belongs to the genus *Flavivirus* under family *Flaviviridae*. JEV is transmitted to humans through the bite of infected *Culex* species mosquitoes (CDC, 2012). However, there is no effective antiviral therapy although a few highly effective vaccines are licensed for human use. Thus the search for antiviral products becomes very important. Virtual screening has repeatedly proven to be useful to meet the special challenges of antiviral drug discovery.

The open reading frame (ORF) of JEV genome translates into a large polyprotein containing 10 proteins. The N- terminal of one fourth of polyprotein encodes structural proteins- Capsid (C), Membrane glycoprotein (prM) and Envelope (E) and non structural (NS) proteins (NS1-NS2-NS2B-NS3-NS4A-NS4B-NS5). NS3 plays an important role in viral genomic RNA replication and viral precursor polyprotein processing. NS3 of JEV is a multifunctional protein which comprises of 619 amino acid residues. NS3 serves as a reservoir for viral proteins during virus assembly (Yamashita *et al.*, 2008). It possesses enzymatic activities of serine protease, helicase and nucleoside 5'-triphosphatase (NTP). One third of the N terminal end of NS3 has a serine protease

activity that participates in the processing together with a cofactor protein NS2B. The NS3 protein also has catalytic domain for helicase, NTPase, as well as 5'-terminal RNA triphosphatase activities in the two-third of the C-terminal end.

Helicase are grouped into three super families and one family based on several conserved motifs (Gorbalenya & Koonin, 1993). Motif I, also known as Walker A, is a phosphate binding P-loop. Motif II, referred to as Walker B has a Magnesium binding aspartic acid loop. Both the motifs exist in all helicase superfamilies (Koonin, 1991). Helicase can be further classified into DEAD/H, DExD, and DExx subfamilies based on the sequence of motif II (Luking *et al.*, 1998). The flavivirus helicase is a member of the DEAH/D box family within helicase super family 2 (Wu *et al.*, 2005). NS3 Helicase/ NTPase is essential for the viral life cycle and this structure motif could be an ideal target for the development of novel potent therapeutic regimen (Chiou *et al.*, 2003). Yamashita T *et al.* (2008) examined three- dimensional structure of JEV helicase/ NTPase domain (amino acid residues 171-619). JEV NS3 helicase comprises of three domains. The domain I (amino acid residues 171–328) contains 4 α-helices and 5 β-sheets. Domain I have two ATP- binding motifs, Walker A and Walker B. Domain II (amino acid residues 329- 482) comprises of 3 α-helices and 8 β-strands. An arginine finger in motif VI in domain II is thought to be crucial for Nucleoside triphosphate (NTP) hydrolysis. Domain III (amino acid residues 483-619) comprises of 7 α-helices and 2 β-strands (Yamashita *et al.*, 2008).

Several studies have been conducted with novel drug inhibitors of JEV in recent times. Flavonoids namely (Kaempferol, Daidzein) and plant secondary metabolite like 4- hydroxyl panduratin A have proved to be effective inhibitors against JEV (Zhang *et al.*, 2012; Seniya *et al.*, 2013). Virtual screening of 4- hydroxypanduratin A showed anti viral activity against JEV NS2B/NS3 protease (Seniya *et al.*, 2013). Besides these compounds, antibiotic group compounds (Aminoglycosides and Tetraycyline) have shown promising effects against flaviviral infections. Broad spectrum antibiotics like Minocycline hydrochloride inhibited JEV and West Nile virus (WNV) induced infection in *in vivo* and *in vitro* experiments (Mishra & Basu, 2008; Michaelis *et al.*, 2007). Antiviral efficacies of two antibiotics (Rolitetracycline and Doxycycline) were found against Dengue virus (DENV) type 2 in a computer simulation that subsequently tallied with *in vitro* experiments (Yang *et al.*, 2007). Since the drugs were found to be effective against other flaviviral etiologies (DENV and WNV), we hypothesized that the drugs could act against JEV as well.

Therefore, docking was performed using iGEMDOCKv.2.1 (Generic Evolutionary Method for Molecular Docking) to study the possible candidate compounds against JEV (Yang *et al.*, 2004). This software is a graphic automatic drug discovery system that involves post screening analysis with pharmacological interactions, which could be used for integrating docking of the target protein NS3 with selected drug compounds. The drugs were retrieved from NCBI PubChem Compound database for docking at specific sites within NS3 helicase/NTPase domain of JEV protein. The selected drugs were then screened; post- analysis and visualization of JEV NS3 helicase domain with compounds were performed. Based on docked conformations of testing compounds, we have proposed a model for the inhibition of JEV NS3 protein. The NS3 protein seems to be a therapeutic drug target. Our findings suggest candidate compounds which could be further explored as therapeutic remedy against JEV.

MATERIAL AND METHODS

Preparation of target protein and ligand selection

JEV NS3 helicase/NTPase structure (PDB ID: 2Z83) was retrieved from RCSB Protein Data Bank (PDB). The molecule was prepared for docking protocol initiation. The protein crystal structures extracted from PDB was prepared for docking with ligands. The structures were opened using Python Molecule Viewer 1.5.6. All the water

molecules were removed, missing residues were added, errors in residues were fixed and hydrogen atoms were added. Kollman charges were added, Gasteiger charges were computed and then the modified structures were saved in PDB format (.pdb). The modified 3D-structure of the protein was opened in Swiss PDB Viewer 4.10 for minimization of energy, identification of established receptor sites and determination of Ramachandran Plot to analyze the stability of the structure. Selection of novel sites for receptor binding was made through Swiss PDB Viewer after analysis of virtual screening results.

For ligand preparation, 10 candidate compounds were selected for the study. The group of compounds selected belonged to Aminoglycoside and Tetracycline groups. Two standards, Ribavirin, a nucleoside antimetabolite and Minocycline hydrochloride, a broad spectrum antibiotic were taken as standard drugs in our all experiments. All candidate compounds were retrieved from NCBI PubChem compound database. The compounds were obtained in MDL MOL format (.sdf) which depicted 3D conformations. Hydrogen atoms and Gasteiger charges were added into the ligands by using UCSF Chimera 1.8 standalone (Pettersen *et al.*, 2004).

Docking Screening

iGEMDOCKv2.1 software provides a graphical environment for recognizing pharmacological interactions and dock screening. Docking of entire NS3 helicase domain with library of 10 compounds was performed. The integrated docking, screening, post- analysis and visualization were performed using RasMol (RasWin Molecular Graphics) software (Sayle *et al.*, 1995).

Docking analysis

iGEMDOCK v2.1 was used to perform molecular dockings. The core of iGEMDOCK v2.1 is the GEMDOCK (Kaladhar, 2012). The program utilizes a Generic Evolutionary Method for molecular docking and an empirical scoring function. Using iGEMDOCK v2.1, the predicted poses generated were directly visualized by a molecular visualization tool and analyzed by post-analysis tools.

Post – processing of docking results and scoring function

iGEMDOCK v2.1 provides the post-analysis tools by using k-means and hierarchical clustering methods based on docked interactions and atomic compositions. The pharmacological scoring function of the docked pose is given as:

$$E_{pharma} = E_{GEMDOCK} + E(E)_{pharma} + 2E(H)_{pharma} + 0.5\ E(V)_{pharma}$$

where $E_{GEMDOCK}$ is the docked energy of GEMDOCK and E (E)pharma, E (H)pharma and E (V)pharma are the pharmacological scores of electrostatics, hydrogen- bonding and Vander Waal's interactions respectively.

Drug susceptibility evaluation on JEV propagation

Mammalian BHK- 21 host cells were cultured at 37°C with 5% CO_2 in minimum essential medium (MEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 5% tryptose phosphate broth (TPB) (Sigma). The drug susceptibility was evaluated by virus yield reduction assay. Brefldin A and Demeclocycline were not tested as both these drugs showed cytotoxicity at minimum drug concentration. Cells were pre-treated with various concentrations of drugs – Chlortetracycline, Doxycycline, Gentamicin, Kanamycin, Minocycline, Rolitetracycline, Tetracycline or Ribavarin (0 - 100 µg/ml) for 24 h prior to infection. BHK 21 cells were inoculated with JEV strain P20778 at a multiplicity of infection (MOI) of 0.1. Following a 1 h virus incubation period, the medium was removed and infected cultures were incubated with medium containing respective concentrations of drugs. At 48 h post-infection, cultures were deep frozen at –80°C. After thawing of the cultures, infectious virus titres were determined for progeny virus yields by standard plaque

titrations. All the experiments were run in triplicate. Percentage inhibitions of plaques were determined using the following formula:

$$\% \text{ inhibition} = \frac{\textit{Number of plaques in virus control} - \textit{number of plaques in drug treated}}{\textit{Number of plaques in virus control}} \times 100$$

The antiviral activity was expressed as 50% inhibitory concentration (IC_{50}) of the compound, i.e. concentration of the compound required to inhibit viral plaques by 50% as compared to virus control (Michaelis *et al.*, 2007; He *et al.*, 2011; Sebastian *et al.*, 2008).

RESULTS

Docking of NS3 helicase/NTPase receptor (Fig. 1) with antibiotic compounds (Fig. 2) was performed. The compounds were arranged in increasing energy or having a low binding affinity (Table 1). Docking analysis showed that Kanamycin (carbohydrate based antibiotic compound that belongs to Aminoglycoside category) and Tetracycline (derivatives of polycyclic naphthacene carboxamide category) group compounds (mainly Rolitetracycline and Doxycycline) showed minimum binding energy that indicated a strong binding affinity between the ligand moieties and viral protein. Kanamycin showed the highest molecular interaction energy. The hierarchical model illustrating the clustering of ligand and protein interactions are given in Fig. 3. The docking conformations of the ligands are visualized in Fig. 4. Majority of bond interactions were intermolecular Vander waals bond {strength (KJ/Mol) – 0.4–4.0 and distance (NM) – 0.3-0.6} and a few hydrogen bonds {strength (KJ/Mol) – 12-30 and distance (NM) – 0.3} (Than). Majority of vander Waals contacts are formed between carbon, oxygen and nitrogen atoms. These three atoms resulted in 15 potential combinations which covered all vander Waals contacts between protein and compounds. The major interacting residues present in NS3 helicase/NTPase which displayed conserved interaction were Lys^{200} (side chain), His^{268} (main and side chain), Asp^{291} (side chain), Gln^{457} (side chain), Glu^{491} (side chain), Asp^{542} (main chain and side-chain), Leu^{543} (main

Figure 1. Crystal structure of NS3 helicase/NTPase of JEV (PDB-ID: 2Z83) showing the Chain A of the protein

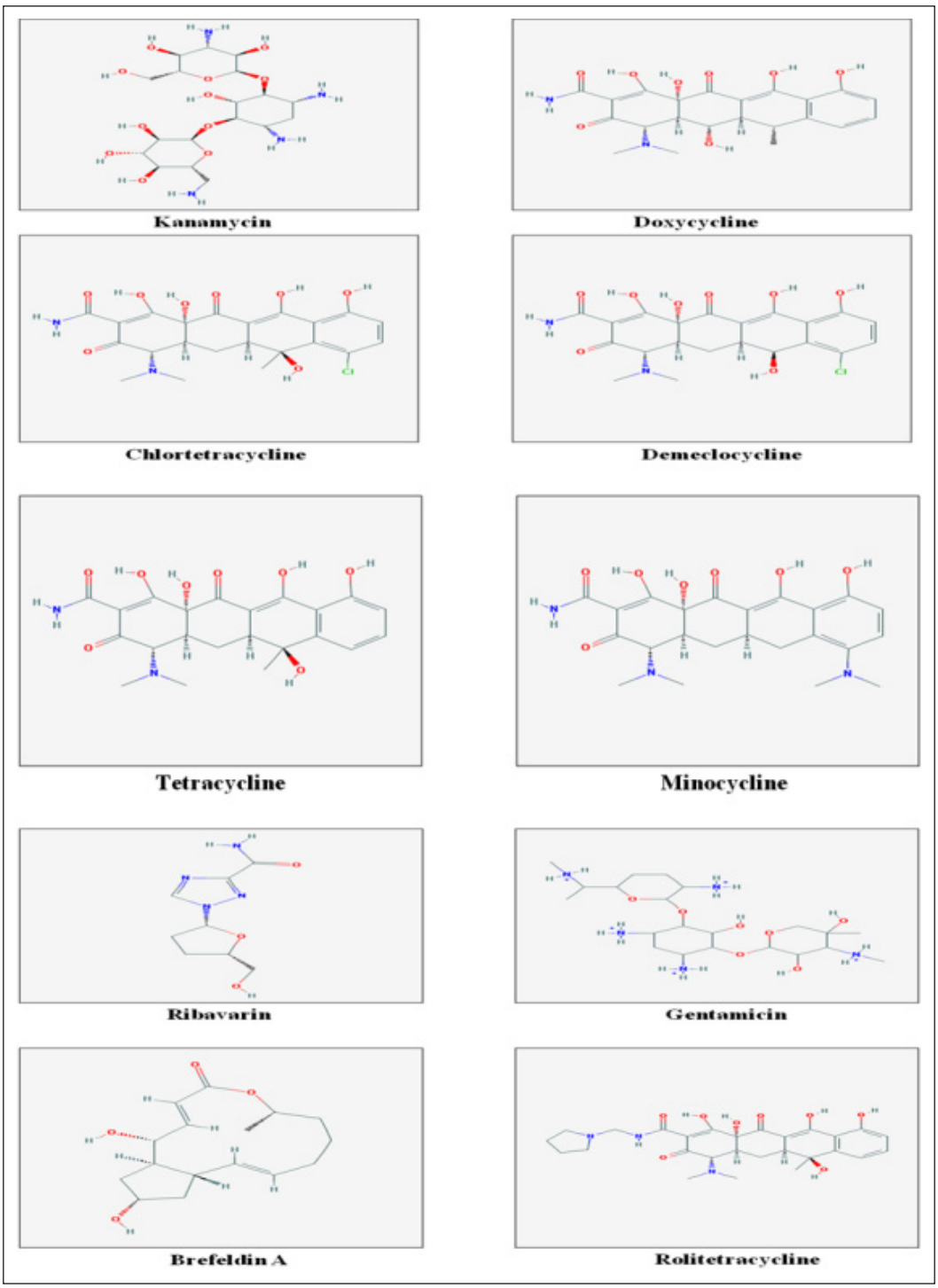

Figure 2. 2-dimensional structures of 10 chemical compounds obtained from NCBI-PubChem Compound database to be used as ligands for screening and docking

Table 1. Energy of fitness of the different ligands with NS3 helicase/ NTPase in increasing order of energy

Compound	Energy (Total) (KJ/mol)	VDW (Van der Waals) Interaction	H Bond (Hydrogen bonding)	Elec (Electrostatic energy)
2Z83(mod)-Kanamycin	-147.367	-147.367	0	0
2Z83(mod)-Rolitetracycline	-124.552	-84.2985	-40.2532	0
2Z83(mod)-Doxycycline	-121.816	-121.816	0	0
2Z83(mod)-Demeclocycline	-118.599	-92.3541	-26.245	0
2Z83(mod)-Chlortetracycline	-116.973	-116.973	0	0
2Z83(mod)-Minocycline	-115.024	-94.8825	-20.1417	0
2Z83(mod)-Brefeldin A	-86.9253	-64.8397	-22.0856	0
2Z83(mod)-Ribavarin	-75.8248	-75.8248	0	0
2Z83(mod)-Gentamicin	-58.9329	-58.9329	0	0
2Z83(mod)-Tetracycline	117.823	130.609	-12.7856	0

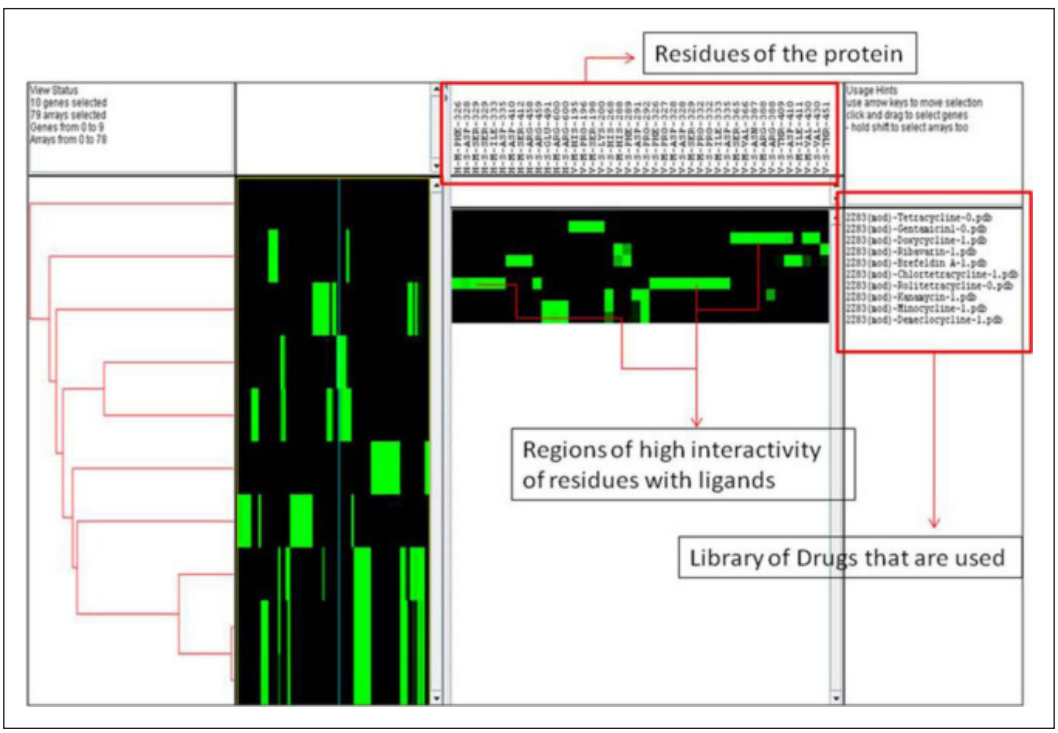

Figure 3. Java Tree View of the 10 ligands and the interacting residues of the protein obtained after virtual screening analysis using iGEMDOCKv2.1

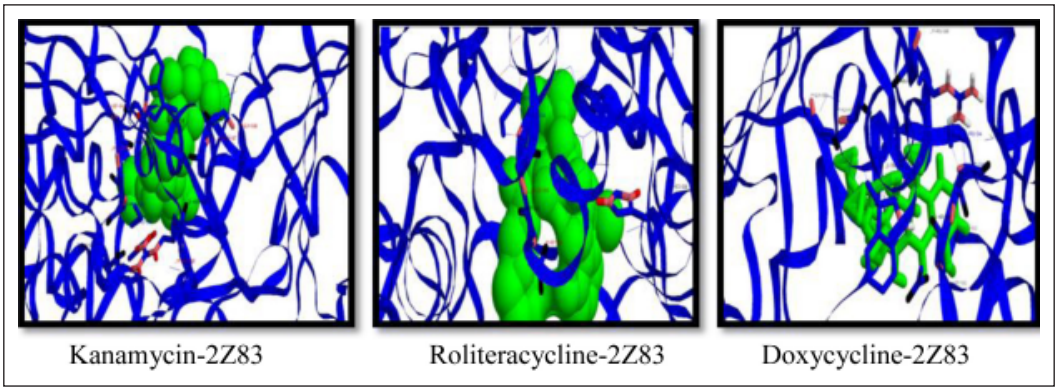

Figure 4. The three docking conformations (from left to right) of NS3 helicase/ NTPase (2Z83) with the 3 best ligands in terms of energy. The conformations are generated on the basis of total pharmacological energy calculated using the pharmacological scoring function of iGEMDOCKv2.1. (All the ligands are coloured in green, carbon atoms in blue, oxygen atoms in black and nitrogen atoms in pink and hydrogen atoms in red)

chain), Val^{545} (main chain), Arg^{600} (main chain and side chain), Val^{601} (main chain), Asp^{604} (side chain), Gln^{606} (main chain and side-chain), Ala^{607} (main chain and side chain) and Trp^{610} (side chain). All these residues were found to interact with the drugs through Vander Waal's forces. Vander Waal's force was the dominant part that contributed to 69% of binding energy. However, Phe^{326} (main chain), Asp^{328} (side chain), Ser^{329} (main chain and side chain), Ile^{333} (main chain) and Asp^{335} (side chain) interact with NS3 by hydrogen bond. Analysis of hierarchical dendrogram showed that bonds were formed in all the three domains- I (18), II (26) and III (13). Amino acid residues in motif II were shown to be essential for RNA helicase activity (Tsai, 1997; Utama *et al.*, 2000). Amino acid of Lys^{200} in walker A motif interacts with phosphates of ATP. The side chain of Lys^{200} plays an important role in the electrostatic interaction with the substrate in NTP hydrolysis with an adequate charge and length to fit with substrate. Also, Gln^{457} of motif VI present in ATP- binding pocket of JEV helicase participate in enzymatic activities of JEV helicase by interacting with the substrate. Amino acids in motifs I and VI participating in an ATPase/helicase activity are important for viral replication as was reported in JEV and DENV (Yamashita *et al.*, 2008; Utama *et al.*, 2000).

Treatment with varying concentrations of drugs resulted in an efficient reduction of progeny virus titers in infected BHK-21 cells. Table 2 shows IC_{50} values of compounds that showed inhibition against JEV *in vitro*. Kanamycin (IC_{50}- 70 µg/ml), Rolitetracycline (IC_{50}- 76 µg/ml) and Doxycycline (IC_{50}- 22 µg/ml) inhibited plaque formation which demonstrated effectiveness against JEV induced infection.

Table 2. IC_{50} of the antibiotic compounds

Serial No	Compound	IC_{50} (µg/ml)± SD
1	Rolitetracycline	76 ± 25
2	Tetracycline	74 ± 33
3	Kanamycin	70 ± 15
4	Gentamicin	53 ± 9
5	Chlortetracycline	50 ± 7
6	Doxycycline	22 ± 1.2
7	Brefldin A	Not done*
8	Demeclocycline	Not done*
Standard drugs		
9	Minocycline	34 ± 9
10	Ribavirin	20 ± 2

* - IC_{50} could not be calculated as both the drugs showed cytotoxicity at minimum concentration range.
SD - Standard deviation

DISCUSSION

We found that the candidate antibiotic compounds had shown bond interactions with JEV NS3 helicase/NTPase protein. Aminoglycoside- Kanamycin and Tetracyclines- Rolitetracycline and Doxycycline showed the most promising results. Interacting ligands docked with minimum energy contributed to highly stable conformation. Bonds were formed in all three domains of NS3 helicase. Maximum bonds were formed in domain II. Few prominent bonds were formed with amino acid residues in motifs I and VI that played a vital role in viral replication (Yamashita *et al*., 2006; Utama *et al*., 2000). Docking showed that the candidate drug compounds were effective inhibitors against JEV by a probable process of acting against viral replication of JEV. Kanamycin, Rolitetracycline and Doxycycline interacted with Lys^{200} and Gln^{457} amino acid residues of the NS3 helicase among other interactions. The binding affinity of these three drugs showed higher binding affinity than the two standards taken in our experiment- Ribavirin and Minocycline was observed. Thus it is seen that the candidate compounds were better inhibitors of JEV NS3 helicase/NTPase protein compared to the standards. The study also indicates that the drugs have acted against viral replication of JEVas the probable mode of action. We further performed biological assay validation in a cell culture system to assess whether the compounds obtained by docking could indeed affect the propagation of JEV replication as exhibited *in silico*. Varying concentrations of the compounds were added separately to the cultures of BHK-21 cells. If the compounds bind to the NS3 proteins as predicted, they may interfere with the interactions with NS3 protein, causing inhibition of viral replication. This inhibition will reduce JEV infection in BHK-21 cells. A plaque is formed due to every successful infection; the number of plaques on the assay plate indicates the number of infection events. IC_{50} values exhibited plaque inhibition, which demonstrated effectiveness against JEV induced infection. However, the *in vitro* results were slightly different with the *in silico* results. Kanamycin, Rolitetracycline and Doxycycline were the best active compounds in *in silico* screening. However, these three compounds were the 3^{rd}, 1^{st} and 6^{th} best compounds inhibiting the virus growth. This data does not exactly match with the *in silico* results. NS3 protein plays an important role in the viral genomic RNA replication and viral precursor polyprotein processing. The contribution of NS3 inhibition to the actual inhibitory activity of the compounds may be attributed to these facts. Kanamycin belongs to the Aminoglycosides group. It acts as a universal RNA binder. The superior binding property exhibited by Kanamycin *in silico* may be ascribed to its 2 Deoxy streptamine (DOS) ligand molecule (Schwab., 2006). Rolitetracycline and Doxycycline belong to Tetracycline group. The hydroxyl group of the main tetracycline ring system is a source of radical oxygen species (ROS) that irreversibly damage RNA, DNA and protein macromolecules (Fuoco, 2012). In another study, the functional groups of the tetracycline structure showed anti- Dengue activity (Yang *et al*., 2007). Thus, it could be stated that the structures of Aminoglycosides and Tetracyclines are involved in interaction with the viral molecule. These interactions caused the NS3 protein inhibition, decreasing the JEV propagation.

Therefore, our study suggests a new possible therapeutic regimen using the antibiotic compounds against JEV infection and other flaviviral etiologies which attain further importance as there is no effective antiviral therapy for treating JEV infection as yet.

Acknowledgments. Rashmee Topno is supported by an ICMR SRF fellowship programme. The authors are grateful to Dr. Pritom Chowdhury and Ms. Purbita Chowdhury for the assistance received during the experimental work.

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