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IN SILICO DRUG DISCOVERY ON DENGUE HEMORRHAGIC FEVER USING TETRACYCLINE ANALOGUES

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ABSTRACT

Dengue hemorrhagic fever is caused by four different serotypes of Dengue virus (DEN-1, DEN-2, and DEN-3, DEN-4). Dengue virus comes under the family flaviviridae. The Dengue infection usually shows severe head ache, fever, rashes all over the body and finally leads to death. Dengue hemorrhagic fever has high motality rate. Tetracycline is found to inhibit the activity of envelope protein during virus entry. The analogues of tetracycline are drawn using ISIS Draw. The energy of those analogues is calculated by using ChemDraw. The docking studies are analyzed and concluded a new molecule is found to have lowest energy potential during docking.

KEYWORDS

Tetracycline derivatives, Dengue virus, Flavivirus, Hemorrhagic Fever, E-protein

INTRODUCTION

Flaviviruses are a major cause of infectious disease in humans. Dengue virus causes an estimated 50 million cases of febrile illness each year, including an increasing number of cases of hemorrhagic fever. West Nile basin to the Western Hemisphere, now causes thousands of sporadic cases of encephalitis annually. Despite the existence of licensed vaccines, yellowfever, Japanese encephalitis and tick-borne encephalitis also claim many thousands of victims each year across their vast endemic areas. Antiviral therapy could potentially reduce morbidity and mortality from flavivirus infections, but no effective drugs are currently available.

DENGUE VIRUS

Dengue (DF) and dengue hemorrhagic fever (DHF) are caused by one of four closely related, but antigenically distinct, virus serotypes (DEN-1, DEN-2, DEN-3, and DEN-4), of the genus *Flavivirus*. Infection with one of these serotypes provides immunity to only that serotype for life, so persons living in a dengue-endemic area can have more than one dengue infection during their lifetime. DF and DHF are primarily diseases of tropical and sub tropical areas, and the four different

dengue serotypes are maintained in a cycle that involves humans and the *Aedes* mosquito. However,

Aedes aegypti, a domestic, day-biting mosquito that prefers to feed on humans, is the most common *Aedes* species. Infections produce a spectrum of clinical illness ranging from a nonspecific viral syndrome to severe and fatal hemorrhagic disease. Important risk factors for DHF include the strain of the infecting virus, as

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well as the age, and especially the prior dengue infection history of the patient.

- Each serotype provides specific lifetime immunity, and short--immunity
- All serotypes can cause severe and fatal disease
- Genetic variation within serotypes
- Some genetic variants within each serotype appear to be more virulent or have greater epidemic potential

Each virus serotype elicits specific lifetime immunity against the same (homologous) serotype, as well as short-term cross-immunity against the other three serotypes, which may last several months. All four serotypes can cause severe and fatal disease. There is genetic variation within each of the four serotypes, and some genetic variants of each serotype appear to be more virulent or have greater epidemic potential.

Genome structure

The mature flavivirus virion is smooth and spherical, with a diameter of 500Å. The genome is packaged by the viral capsid protein (C) in a host-derived lipid bilayer in which 180 copies of the envelope protein (E) is embedded.The E protein is initially complexed with the precursor membrane protein (prM) during assembly of the virions in the endoplasmic reticulum forming immature particles. The immature particles are transported to trans-Golgi compartment where they undergo maturation by the cellular serine protease, furin, which mediates cleavage of prM to M resulting in homodimerization of E protein to form fusion-competent mature particles before release into circulation. The single-stranded positive-sense RNA genome contains a single long open reading frame flanked by 5_- and 3_-untranslated regions, which have secondary structures that are essential for the initiation of translation and for replication. The 5_ end of the genome has a type 1 cap, but the 3_ end lacks a poly-A tail. Translation of the genome by the host cell machinery produces a polyprotein comprising the viral structural and non-structural proteins that are required for replication and assembly of new virions.

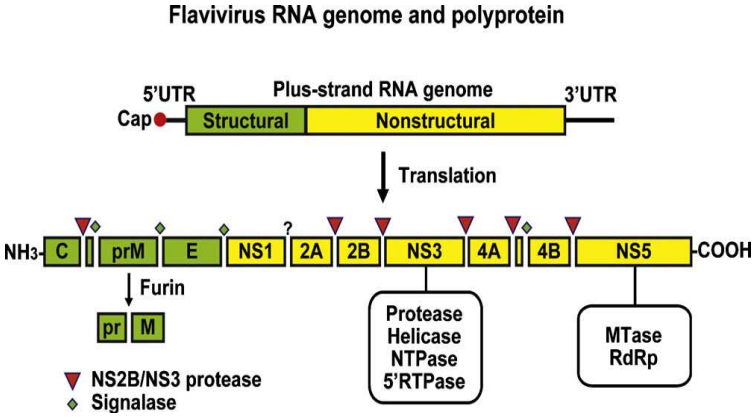

Figure 1

Flavivirus RNA genome and polyprotein

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Signs and symptoms

The disease manifests as a sudden onset of severe headache, muscle and joint pains (myalgias and arthralgias—severe pain that gives it the nick-name *break-bone fever* or *bonecrusher disease*), fever, and rash. The dengue rash is characteristically bright red petechiae and usually appears first on the lower limbs and the chest; in some patients, it spreads to cover most of the body. There may also be gastritis with some combination of associated abdominal pain, nausea, vomiting, or diarrhea. Some cases develop much milder symptoms which can be misdiagnosed as influenza or other viral infection when no rash is present. Thus travelers from tropical areas may pass on dengue in their home countries inadvertently, having not been properly diagnosed at the height of their illness. Patients with dengue can pass on the infection only through mosquitoes or blood products and only while they are still febrile. The classic dengue fever lasts about six to seven days, with a smaller peak of fever at the trailing end of the disease (the so-called *biphasic pattern*). Clinically, the platelet count will drop until the patient's temperature is normal. Cases of DHF also show higher fever, variable haemorrhagic phenomena, thrombocytopenia, and haemoconcentration. A small proportion of cases lead to dengue shock syndrome (DSS) which has a high mortality rate. DHF combined with a cirrhotic liver has been suspected in rapid development of hepatocellular carcinoma (HCC). Given that the DEN virus is related to the Hepatitis C virus, this is an avenue for further research as HCC is among the top five cancerous causes of death outside Europe and North America. Normally HCC does not occur in a cirrhotic liver for ten or more years after the cessation of the poisoning agent. DHF patients can develop HCC within one year of cessation of abuse.

LIFE CYCLE

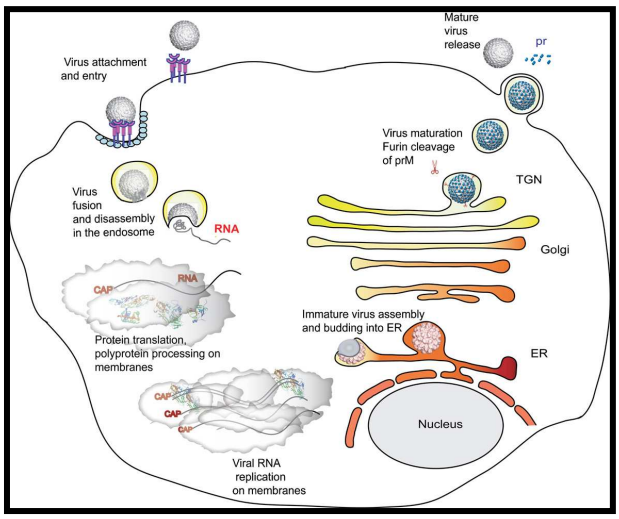

Figure 2.
Life Cycle

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Host cells for flaviviral infection include monocytes, macrophages and dendritic cells. The virus attaches to the cell surface, mediated by the E protein, and enters the cell by receptor-mediated endocytosis (Fig. 2). Low pH in the endosomal compartment triggers fusion of the viral and host cell membrane mediated by structural reorganization of E, which leads to the release of the nucleocapsid and viral RNA into the cytoplasm. Translation of the RNA generates a polyprotein that is co-translationally and post-translationally processed by the virus-encoded serine protease, NS2B/NS3, and by host-encoded proteases, including signalase and furin, to produce the 3 structural proteins and 7 non-structural proteins in the order C-prM-E -NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 .NS3 (70 kDa) and NS5(104 kDa) are the best characterized non-structural proteins, with multiple enzyme activities that are required for viral replication. NS3 has three distinct activities:serine protease together with the cofactor NS2B, required for polyprotein processing; helicase/NTPase activity, required for unwinding the double-stranded replicative form of RNA; RNA triphosphatase, required for capping nascent viral RNA; Mutations that affect each activity impair viral replication . NS5 is the largest and most highly conserved flaviviral protein, with greater than 75% sequence identity across all DEN serotypes. It contains two distinct enzymatic activities, separated by an interdomain region: An *S*-adenosyl methyltransferase (SAM) and an RNA-dependent RNA polymerase (RdRp) NS1 (46kDa) is required for flavivirus replication and presumably involved in negative-strand synthesis by an unknown mechanism. A large deletion in YFV NS1 abolished viral replication but can be complemented in trans by functional expression from Sindbis virus vector .Furthermore, a temperature sensitive mutation (Arg-299) is defective in viral replication at 39°C and fails to accumulate negative strand RNA, but is functional at 32°C, suggesting that NS1 is required for negative strand RNA synthesis NS2A (22 kDa) is a small hydrophobic transmembrane protein that is involved in generation of virus induced membranes during virus assembly .NS4A (16 kDa) is an integral membrane protein which induces membrane rearrangements to form the viral replication complex. NS4B (27 kDa) inhibits the type I interferon response of host cells and may modulate viral replication via its interaction with NS3 Viral RNA replication occurs in the rough endoplasmic reticulum (ER) and in Golgi-derived membranes called vesicle packets (VP). The non-structural proteins and dsRNA are concentrated in the VP, constituting the site of viral RNA synthesis. The newly synthesized viral RNA is extruded in the intermembrane space of the double-membrane VPs, from which it exits into the cytoplasm by an unknown mechanism. Assembly of virus particles occurs in the lumen of the rough ER. The first step in this process is the coating of the newly synthesized viral RNA with the C protein. Next, E and PrM hetero-dimerize and envelope the nucleocapsid, forming an immature virus particle that buds from the RER lumen into the Golgi. However, the mechanism of interaction of the C protein within the nucleocapsid is still not clear. Maturation of virus particles occurs in the trans-Golgi network, where prM is cleaved to M by furin, along with conformational rearrangements of E. This is an essential step for the virus in the transition from fusion-incompetent and non-infectious virus particles to mature, fusion-competent, and infectious virions. The mature particles eventually exit from the host cell by exocytosis.

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TRANSMISSION OF DENGUE VIRUS

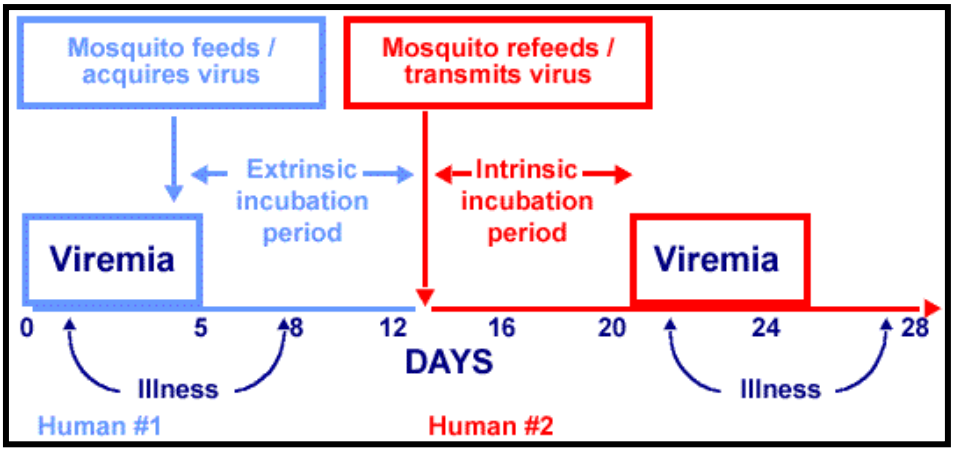

Figure 3
***Transmission of Dengue* Virus**

The transmission cycle of dengue virus by the mosquito *Aedes aegypti* begins with a dengue-infected person. This person will have virus circulating in the blood—a viremia that lasts for about
five days. During the viremic period, an uninfected female *Aedes aegypti* mosquito bites the person and ingests blood that contains dengue virus. Although there is some evidence of transovarial

transmission of dengue virus in *Aedes aegypti*, usually mosquitoes are only infected by biting a viremic person. Then, **within the mosquito**, the virus replicates during an **extrinsic** incubation period of eight to twelve days. The mosquito then bites a susceptible person and transmits the virus to him or her, as well as to every other susceptible person the mosquito bites for the rest of its lifetime. The virus then replicates in the second person and produces symptoms. The symptoms begin to appear an average of four to seven days after the mosquito bite—this is the **intrinsic** incubation period, **within humans**. While the intrinsic incubation period averages from four to seven days, it can range from three to 14 days. The viremia begins slightly before the onset of symptoms. Symptoms caused by dengue infection may last three to 10 days, with an average of five days, after the onset of symptoms—so the illness persists several days after the viremia has ended.

E-PROTEIN AND DRUG

The 26 kDa glycosylated precursor of M protein, prM, is processed from a polyprotein in the ER by the host signalase by cleavages at capsid-prM site at its N terminus and prM-E site at its carboxy terminus. The association of prM with E produces non-infectious, immature virus particles. The arrangement of prM in the prM-E heterodimers in the immature particles protects the fusion loop of E protein from premature fusion. The “immature” particles transit through a low pH environment of the Golgi compartment, and a reversible conformational/morphological change occurs in E protein prior to processing of prM. Cleavage of prM to M by cellular serine protease, furin, in the trans-Golgi network results in an irreversible conformational change in E. The peptide cleaved off

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from prM (pr) is retained on the virion, and is released only after the virion has been secreted out and exposed to neutral pH, thus protecting the E protein from premature fusion (Li et al., 2008; Yu et al., 2008a). The 53 kDa E protein in its mature dimeric form is the major surface component of the virion. In this form, the E protein is competent for cell surface attachment, fusion and virus entry into host cells as described by Virus entry followed by endosomal acidification induces structural changes, in which E rearranges fro 90 homodimers in neutral pH to 60homotrimers in acidic pH. The fusion loop on the DII domain that was buried in the DI/DIII pocket is now exposed in the fusogenic state of E trimer prior to its insertion into the host cell membrane.

In the drug discovery point of view, development of a robust high throughput assay based on protein–protein interactions would be very useful to screen conformational transitions of prM and E review recent advances in understanding the dynamics of the flavivirus E protein and suggest three regions within the protein that could be targeted by antiviral: the _-OG ligand binding pocket, E-protein rafts in the mature virus and E homotrimers. The availability of structural information from immature and mature particles is thus paving the way for rational drug.

A process of virtual screening has been developed based on the observation that conformational rearrangements of the dengue virus (DV) envelope protein are essential for the mediation of viral entry into host cells via membrane fusion limited structural information of drug targets, cellular toxicity possessed by lead compounds, and large amounts of potential leads are the major issues facing the design-oriented approach of discovering new leads. In an attempt to tackle these issues, we have developed a process of virtual screening based on the observation that conformational rearrangements of the dengue virus envelope protein are essential for the mediation of viral entry into host cells via membrane fusion. Screening was based solely on the structural information of the Dengue virus envelope protein and was focused on a target site that is presumably important for the conformational rearrangements necessary for viral entry. Screening is based on molecular docking using structural databases of medical compounds. They successfully identified rolitetracycline and doxycycline significantly inhibited plaque formation, demonstrating their inhibitory effect on dengue virus propagation. Both the compounds are tetracycline derivatives.

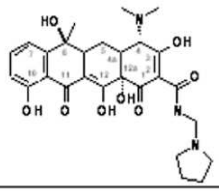
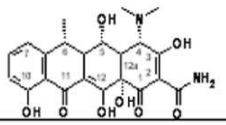
Compound	Structure	Name	IC_{50}
1		rolitetracycline	67.1 µM
2		doxycycline	55.6 µM

Figure 4
Datasets of Tetracycline Derivatives

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MATERIALS AND METHODS

The Protein Data Bank (PDB) is a repository for the 3-D structural data of large biological molecules, such as proteins and nucleic acids. (See also crystallographic database). The data typically obtained by X-ray crystallography or NMR spectroscopy and submitted by biologists and biochemists from around the world, are released into the public domain, and can be accessed at no charge on the internet. The PDB is overseen by an organization called the Worldwide Protein Data Bank, wwPDB.

The PDB is a key resource in areas of structural biology, such as structural genomics. Most major scientific journals, and some funding agencies, such as the NIH in the USA, now require scientists to submit their structure data to the PDB. If the contents of the PDB are thought of as primary data, then there are hundreds of derived (i.e., secondary) databases that categorize the data differently. For example, both SCOP and CATH categorize structures according to type of structure and assumed evolutionary relations; GO categorize structures based on genes.

3.2. ISIS DRAW

ISIS/Draw was a chemical structure 2D drawing program for Windows, published by MDL Information Systems. It is available free of charge for academic and personal use. ISIS/Draw uses its own proprietary file format, with the extension *.skc, and also supports standard chemical file formats such as MDL molfile, Rxnfile, and TGfile. ISIS/Draw is a chemical drawing program somewhat similar to ChemDraw. It has some 3D rotation features and can interface with Rasmol for 3D visualization and rendering. ISIS/Draw also includes structure and reaction validation features and can calculate elementary properties such as formula and molecular weight.

METHODS

Collection of Research data: The data related to the protein target such as their metabolism, regulation and the involvement in the diseases are collected from the reviews and the research articles and journals. The structure of the protein targeted is retrieved from the database Protein Data Bank.

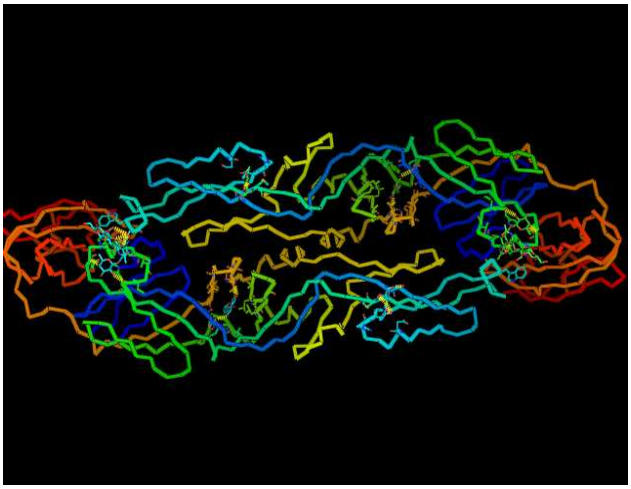

Figure 5
PDB ID: 1oke

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Table 1
Protein Description

Particulars	Description
Molecule	Envelope protein in complex with n-octyl-beta-d-glucoside
Chains	A
Pdb id	1oke
Structural weight	90132.14
Source	Drosophila melanogaster
Experimental mehod	X-ray diffraction
Resolution	2.40
R - value	0.263 (obs.)
R- free	0.294
Ligand component	n-acetyl-d-glucosamine

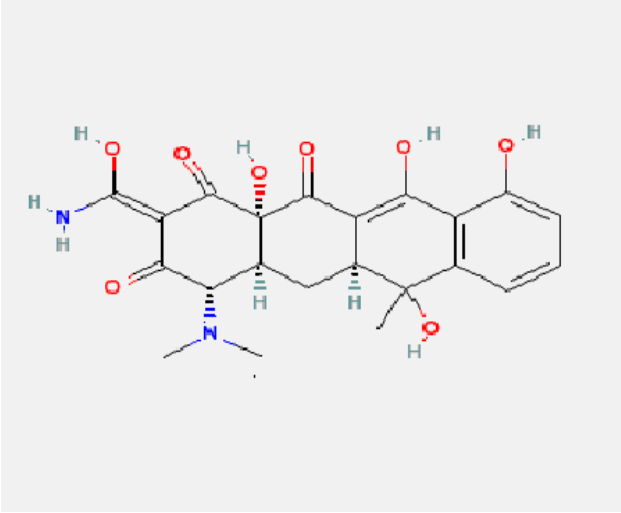

Fig 6.
str.tetracycline derivative Compound ID **5497101**

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Table 2
Energy Minimization result of Ligand

Molecular	444.43456
Molecular	$C_{22}H_{24}N_2O_8$
XLogP3	-1.3
H-Bond Donor	6
H-Bond	10
Rotatable Bond	1
Tautomer Count	168
Exact Mass	444.153266
MonoIsotopic	444.153266
Topological	182
Heavy Atom	32
Formal Charge	0
Complexity	971
Isotope Atom	0
Defined Atom	4
Undefined Atom	1
Defined Bond	1
Undefined Bond	0
Covalently-	1

IUPACName:(2Z,4S,4aS,5aS,12aS)-2-amino (hydroxy) methylidene]-4-(dimethylamino)-6,10,11,12a-tetrahydroxy-6-methyl-4,4a,5,5a-tetrahydrotetracene-1,3,12-trione

CanonicalSMILES:
CC1(C2CC3C(C(=O)C(=C(N)O)C(=O)C3(C(=O)C2=C(C4=C1C=CC=C4O)O)O)N(C)C)O
IsomericSMILES:
CC1([C@H]2C[C@H]3[C@@H](C(=O)/C(=C(\N)/O)/C(=O)[C@]3(C(=O)C2=C(C4=C1C=CC=C4O)O)O)N(C)C)O
InChI=1S/C22H24N2O8/c1-21(31)8-5-4-6-11(25)12(8)16(26)13-9(21)7-10-15(24(2)3)17(27)14(20(23)30)19(29)22(10,32)18(13)28/h4-6,9-10,15,25-26,30-32H,7,23H2,1-3H3/b20-14-/t9-,10-,15-,21?,22-/m0/s1

InChIKey:JYHCQVWYCGHXGP-ONGFDOKGSA-N
Identification of active site and binding pocket:
The active site and the binding pocket is first identified in the target protein to be inhibited. The active site and the binding pocket are identified in four levels.
Level 1 from patent data: Clearly studied the active site and binding pocket from the research data available for the protein. The active site is the place where the nucleotide binds. The binding pocket favorably associates with another chemical entity or compound.

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Map of the active site: C-terminal consists of α - helical domain and N-terminal consists of β - strand domain and bordered by Glycine loop and the hinge. The active site lays in between the aminoacids 260 to 286.

Level 2 from Q-site: The active site is studied clearly from the Q-siteFinder under the URL www.modelling.leeds.ac.uk/qsitefinder/. In the Q-siteFinder either the PDB ID is given or the protein filename Path can be submitted. The server clearly provided the number of active sites present and the residues and the residues number.

Level 3 from Pocket-Finder: The Pocket-Finder is opened using the URL and the PDB ID is provided in the field or the protein itself is loaded using the browse button. The server clearly pictures out the binding pocket of the protein and the residues present within that pocket.

Level 4 from LigandScout: The tool clearly pasteurizes the active site present Hydrogen Bond donor, Hydrogen bond acceptor, and Negative and Positive ionisable area, hydrophobic interaction, Aromatic ring, Metal Binding feature, excluded volume. Ligandscout is software that used for pharmacophore prediction; here the pharmacophore for those eight proteins and this is the result for protein 1oke of E-protein of Dengue virus.

CHEM 3D ULTRA

In chem3D ultra we want to do the energy minimization of the structure. Here I had done the energy minimization by using MM2 and MOPAC.

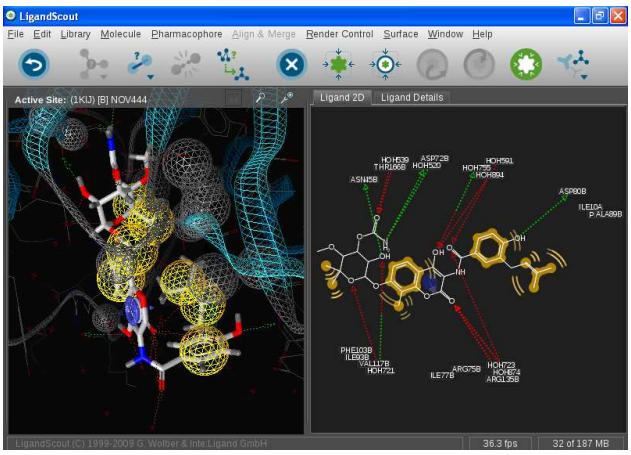

Figure 7.
Pharmacophore prediction through LigandScout

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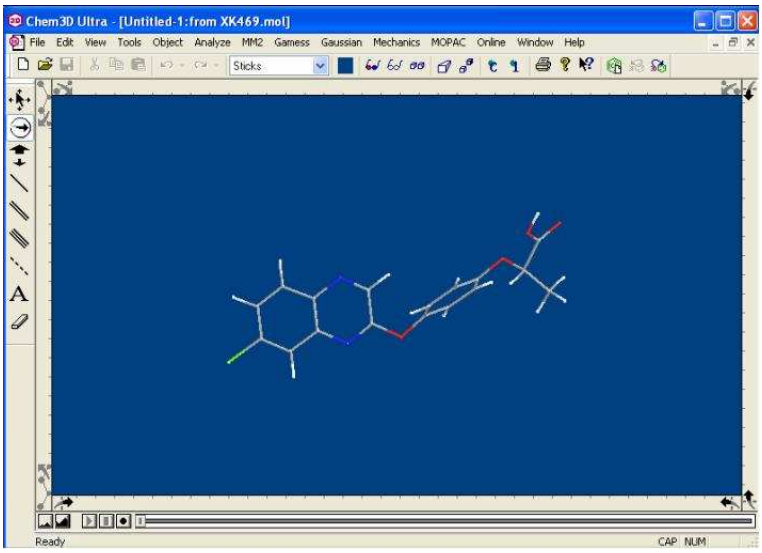

Figure 8.
The tetracycline derivative drawn using Chem3D Ultra

Table 3
Energy minimization done by Chem3D ultra

Final heat of formation	154.83480
Kcal	647.82880 kj
Total energy	-6001.03796 ev
Electronic energy	-45989.33824 ev
Point group	C1
Core-core repulsion	39988.30028 ev
Ionization potential	9.25159
No. Of filled levels	83
Molecular weight	464.449

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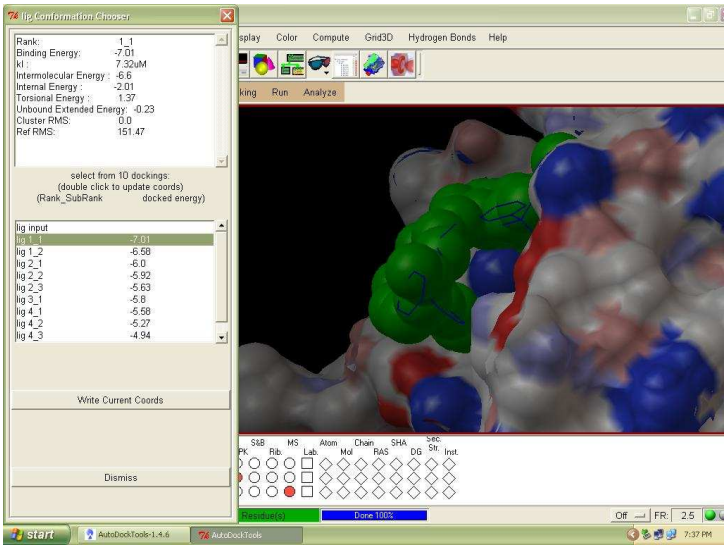

Figure 8.
Structural Based Conformation

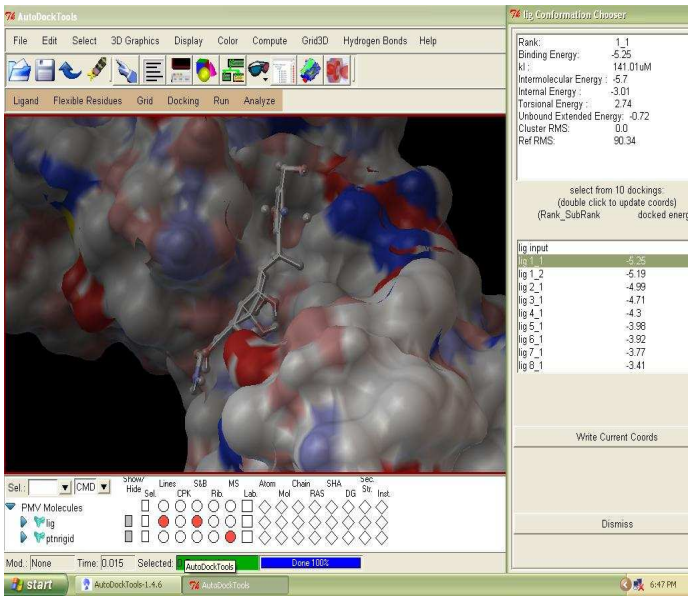

Figure 9
Energy based conformation using AutoDock tool

Auto dock is the software that used to bind the ligand molecule into the protein. The docking with the doxycycline to the envelope protein of dengue virus. The conformation in which the energy is minimized at the range of –8.05. It is concluded that this least conformation changes.

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Table 4
Energy Minimized Value

Properties	Values
Rank	1_1
Binding Energy	-7.01
kI	7.32nM
Intermolecular Energy	-6.6
Internal Energy	-2.01
Torsional Energy	1.37
Unbound Extended energy	-0.23
Cluster RMS	0.0
Ref RMS	151.47

CONCLUSION

We have identified 72 completely conserved amino acid residues in the E protein of major groups of the Flavivirus genus by computational analyses. In the dengue species we have identified 12 highly conserved sequence regions, 186 negatively selected sites, and many dengue serotype-specific negatively selected sites. The flavivirus-conserved sites included residues involved in forming six disulfide bonds crucial for the structural integrity of the protein, the fusion motif involved in viral infectivity, and the interface residues of the oligomers. The structural analysis of the E protein showed 19 surface-exposed non-conserved residues, 128 dimer or trimer interface residues, and regions, which undergo major conformational change during trimerization. Eleven consensuses T (h)-cell epitopes common to all four dengue serotypes were predicted. Most of these corresponded to dengue-conserved regions or negatively selected sites. Of special interest are six singular sites (N (37), Q (211), D (215), P (217), H (244), K (246)) in dengue E protein that are conserved, are part of the predicted consensus T (h)-cell epitopes and are exposed in the dimer or trimer. We propose these sites and corresponding epitopic regions as potential candidates for prioritization by experimental biologists for development of diagnostics and vaccines that may be difficult to circumvent by natural or man-made alteration of dengue virus.

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