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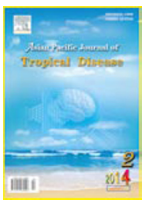

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Analyzing the interaction of a herbal compound Andrographolide from *Andrographis paniculata* as a folklore against swine flu (H1N1)

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ABSTRACT

Objective: To find new bioactive molecules for the treatment of swine flu.
Methods: The present study is an attempt to elucidate inhibition potential of andrographolide and its derivatives along with an associated binding mechanism through virtual screening and molecular docking simulation studies.
Results: Our findings revealed structural conformation changes in 150 loop, secondary sialic acid binding site residues of ACZ97474 {Neuraminidase (A/Blore/NIV236/2009(H1N1)}. Andrographolide have been identified as the highest binging energy of −10.88 Kcal/mol, 3 hydrogen bond interactions (Arg152, Lys150, and Gly197), total intermolecular energy of −12.07 Kcal/mol with bioactivity value (Ki) of 10.59 nmol/L, while the Food and Drug Admistraton approved drug Oseltamivir and Zanamivir have shown 2 and 4 hydrogen bond interactions with binding energies of −6.28 Kcal/mol and −7.73Kcal/mol, respectively, which is higher than andrographolide. The guanidine group of Arg152 has binding affinities to the hydrophilic nature of the inhibitors (−OH and =O groups), as identified by docking of andrographolide (CID: 5318517) on neuraminidase.
Conclusions: Hence, andrographolide has the potential to inhibit neuraminidase activity of H1N1 and may be used as an alternative medicinal therapy for swine flu positive patient. With potent antiviral activity and a potentially new mechanism of action, andrographolide may warrant further evaluation as a possible therapy for influenza.

1. Introduction

Andrographis paniculata (Burm. f.) Nees (Acanthaceae) (*A. paniculata*, *Chuanxinlian*), is a medicinal herb used in many regions such as Taiwan, Mainland China and India to treat liver disorders, colic pain, common cold, and respiratory infections[1-3]. Andrographolide and its derivatives have been widely used for treating respiratory infections in China and India for decades. It contains diterpenoids, flavonoids and polyphenols as the major bioactive components[4]. Andrographolide is the major diterpenoid in *A. paniculata*, making up about 4%, 0.8%~1.2% and 0.5%~6% in dried whole plant, stem and leaf extracts respectively[5-7]. It is also used as a wonder drug in traditional Sidha and Ayurvedic system of medicine as well as tribal medicine in India for multiple clinical applications, since ancient and also been shown to be effective against certain cancers and is an effective purgative. The plant extracts exhibit antityphoid and antifungal, antihepatotoxic, antibiotic, antimalarial, antihepatitic, antithrombogenic, antiinflammatory, anti-snake venom and antipyretic properties to mention a few, besides its general use as an immunostimulating[8-11]. Andrographolide has been tested in different experimental studies on human and animals which proved andrographolide was a safe drug with no harmful side effects[12].

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The swine flu is a severe contagious disease caused by H1N1 virus which leads to respiratory tract infection, other complications such as bronchitis and pneumonia in human. World Health Organization reported 12 787 cumulative confirmed cases and 413 cumulative deaths cases, all caused by H1N1, on 18 Oct 2009[13]. H1N1 contains two surface glycoproteins: hemagglutinin and neuraminidase. Hemagglutinin facilitates the influenza virus to attach to a host cell during the initial infection and viral RNA enters the cell by endocytosis. Neuraminidase cleaves α-ketosidic linkage between the sialic acid (N-acetylneuraminic) and an adjacent sugar residue and spread infection in cells. The amino acid sequence of neuraminidase is coded by the 6th RNA segment and the polypeptide chain and neuraminidase comprises of 470 amino acid residues. The inhibition of neuraminidase is useful in prevention of H1N1 and could serve as potential drug target. Due to development of resistance in many strains of H1N1, the Food and Drug administration approved neuraminidase inhibitor drugs such as Oseltamivir and Zanamivir[14-16]. Moreover, due to several sides effects like nausea, vomiting, abdominal pain and headache, rash and sometimes allergic reactions including anaphylaxis etc, there is a call for new inhibitors against H1N1 influenza A virus with less or no side effects.

The amino acid sequence of neuraminidase [A/Blore/NIV236/2009(H1N1)] (GenBank: ACZ97474.1) is known but the three-dimensional structure is not available[17]. In this study, therefore, we have constructed the 3D structure of ACZ97474 by homology modeling and thereafter taken for interaction study between andrographolide and ACZ97474. The utmost importance in a structure-based drug design is the reliable filtering of putative hits in terms of their predicted binding affinity; which is based on the *in-silico* generated near native protein-ligand configurations. Andrographolide and its derivatives were used in this study to identify inhibitory potential through several receptor-centric computational methodologies for computational modeling of andrographolide and its derivatives as potent inhibitors of neuraminidase protein of H1N1 (ACZ97474).

2. Materials and methods

2.1. Sequence analysis

The protein sequence of ACZ97474 {neuraminidase (A/Blore/NIV236/2009(H1N1)} strain was obtained from Influenza Virus Resource, the official database of the Influenza Virus Resource at the National Center for Biotechnology Information[18]. This protein comprises 421 amino acids. Sequence similarity search with BLAST in Protein Data Bank (PDB) database gives similar proteins 99% identical. Template (pdb ID: 3TI4) was selected for molecular modeling of ACZ97474 which has a good resolution of 1.6 Å. We performed the pairwise alignment of ACZ97474 with 3TI4 as reference using the LALIGN of EMBOSS (Figure 1)[19].

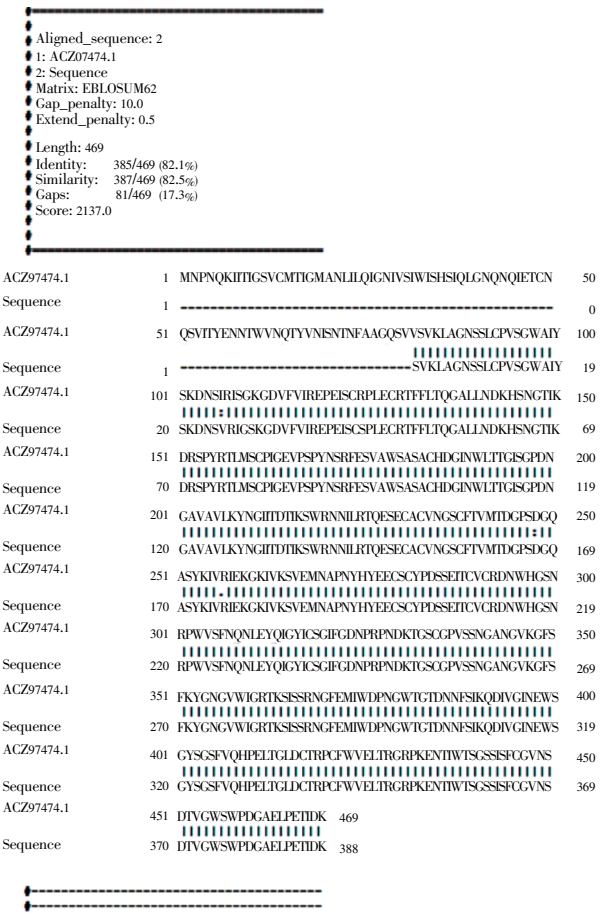

Figure 1. Alignment of ACZ97474 receptor protein sequence with 3TI4 as reference protein.

2.2. Homology model construction

The homology model of the protein (ACZ97474) was built using ModWeb online server[20]. ModWeb is a server for comparative protein structure modelling which depends on the large scale protein structure modelling pipeline, ModPipe, for its functionality[21]. The structural template used to build models in ModPipe was 3TI4 (PDB ID). Sequence-structure matches are established using multiple variations of sequence-sequence, profile-sequence, sequence-profile and profile-profile alignment methods. Significant alignments (E-value better than 1.0) covering at least 30 amino acid residues are selected for modeling. Finally, the resulting models are evaluated

using several model assessment schemes. Model evaluation was performed in PROCHECK v3.4.4 producing plots which were analyzed for the overall and residue-by-residue geometry[22]. Ramachandran plot provided by the program PROCHECK assured very good confidence for the predicted protein[23]. Nevertheless, PROCHECK assured the reliability of the structure and the protein was subjected to Verify3D available from NIH MBI Laboratory Servers[24].

2.3. Ligand Binding Site Prediction

In-silico prediction of binding site was done for the ACZ97474 in neuraminidase [A/Blore/NIV236/2009(H1N1)] using CASTp, Q-Site finder and compared by extensive literature search[25-27]. Best active sites were selected by comparing prediction of CASTp algorithm and Q-Site Finder. The binding sites, active sites, surface structural pockets (accessible), interior cavities (inaccessible), shape (alpha complex and triangulation), area and volume (solvent and molecular accessible surface) of each pockets and cavities of proteins were identified and measured by CASTp method. The number, area, circumference of mouth openings of each pocket in solvent and molecular accessible surface was determined by CASTp[25]. Ligand binding site on a protein was predicted by Q-site finder. Binding of hydrophobic probes to proteins are used to determine binding energy of the probe clusters on the protein and probe clusters with favourable binding energy are arranged in an order[26]. Pockets on the surface of the protein were detected by Pocket finder method which scans the probe radius (1.6 Å) with a grid resolution 0.9 Å, cubic diagonals and ligands along the proteins[28].

2.4. Ligand preparation

An initial dataset of 140 andrographolide analogues was collected from NCBI PubChem compound database and virtually screened on the basis of Lipinski's rule of 5 in which several different ring systems are represented[29-30]. The ligands were converted into PDB coordinate files using OpenBabel software (<www.openbabel.org/>). Ligands were prepared by adding hydrogen bonds and neutralization of charged groups. The optimized ligands were subsequently docked against NS2B using Autodock4.2[31]. Each of these compounds had associated *in vitro* bioactivity values (IC50 values reported in nmol/L) against neuraminidase [A/Blore/NIV236/2009(H1N1)] strain. In order to check the reliability of the geometry obtained, we compared the structural parameters of the andrographolide with theoretical and experimental values from the literature.

2.5. Docking of ligands

In order to understand the binding pattern and mechanism of andrographolide herbal derivatives towards the inhibition of H1N1, docking of all herbal compounds to the ACZ97474 receptor was performed using Autodock4.2[31]. After ensuring that protein and ligands are in correct form for docking the receptor-grid files were generated using grid-receptor generation program using van der Waals scaling of the receptor at 0.6. The default size was used for the bounding and enclosing boxes. The ligands were docked initially using the "standard precision" method and further refined using "xtra precision" (Lamarckian genetic algorithm) with standard docking protocol. Ten independent docking runs were carried out for each ligand and results were clustered according to 1.0 Å RMSD criteria. Best ligands were selected on the basis of H-bonds formation, hydrophobic interactions and minimum binding energies obtained after docking. A single best conformation for each ligand was considered for further analysis.

3. Results

The atomic coordinates of ACZ97474 neuraminidase Influenza A virus[A/Blore/NIV236/2009(H1N1)] was not available in PDB, hence, to develop a protein model was necessary. The final model, which we took for further analysis, consisted of 469 amino acid residues with a resolution of 2 Å. Both PROCHECK and Verify3D softwares have been used to check the quality of the modeled protein structure. Ramachandran plot obtained from the program PROCHECK, which checks the stereochemical quality of a protein structures, producing a number of postscript plots analyzing its overall and residue-by-residue geometry, assured the reliability of the modeled protein with 83.3% residues in most allowed region and 10.8% in additional allowed region. There were none residues in disallowed region and only 0.9% in generously allowed region (Figure 2). The assessment with Verify3D, which derives a "3D-1D" profile based on the local environment of each residue, described by the statistical preferences for: the area of the residue that is buried, the fraction of side-chain area that is covered by polar atoms (oxygen and nitrogen) and the local secondary structure, also substantiated the reliability of the three-dimensional structure. Active sites were identified with reference to the studies done on 3TI4 using CASTp, Q-Site finder and further verified from available literature (Table 1). Here, we combined

the results obtained from a standard docking protocol and then investigated efficiency for andrographolide derivatives and other four herbal compounds imperatorin, andrographolide, epigallocatechin and arabinoxylan from *Angelica archangelica, A. paniculata, Green tea* and *Hyphomycetes mycelia*, respectively (Figures 5–8).

Table 1

Active sites in neuraminidase (ACZ97474) of A/Blore/NIV236/2009(H1N1) strain.

From literature	CASTp	Q-Site finder
Arg118	Arg118	Arg118
Asp151	Asp151	Asp151
Arg152	Arg152	Ser367
Ile222	Ile223	Arg368
Arg224	Arg225	Trp399
Glu276	Glu278	Ser400
Arg292	Arg293	Ile427
Arg371	Arg368	Arg430

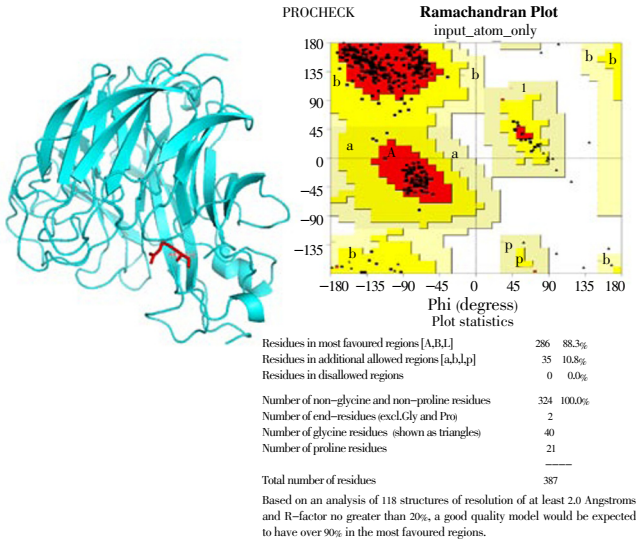

Figure 2. Predicted structure and Ramachandran plot of neuraminidase protein (ACZ97474).

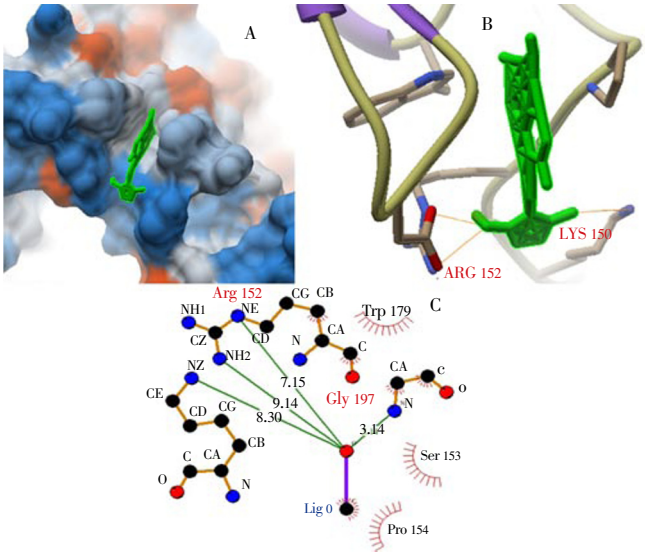

Figure 3: Dcoked C20H30O5 (CID: 29927575) into active site residues of ACZ97474, (A) – Docked Ligand in binding surface, (B) & (C) – Ligand intraction with active site residues in NA using Chimera and Ligplot, respectively

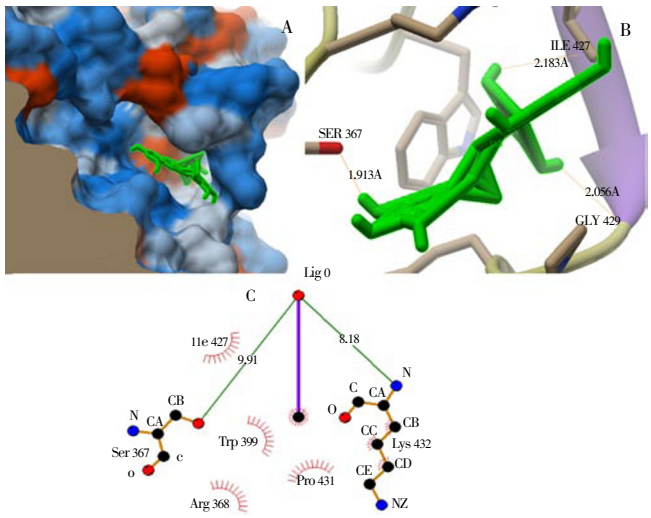

Figure 4. Dcoked $C_{20}H_{30}O_5$ (CID: 6857767) into active site residues of ACZ97474. A: docked ligand in binding surface; B and C: ligand intraction with active site residues in neuraminidase using Chimera and Ligplot, respectively.

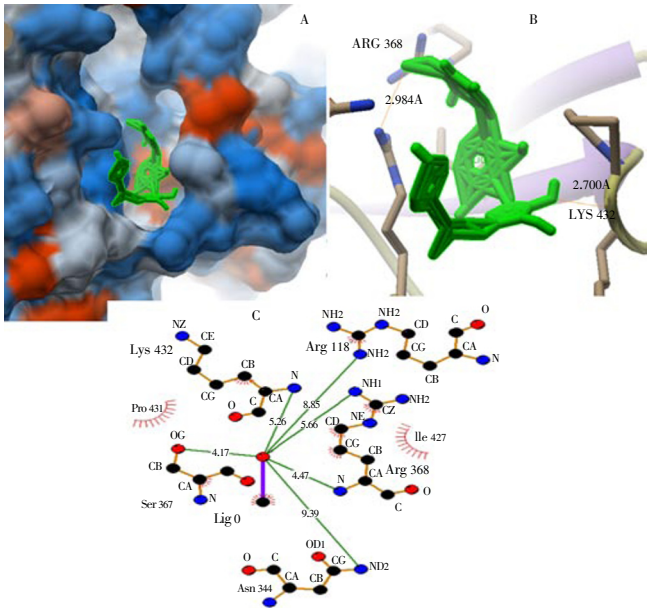

Figure 5. Dcoked andrographolide (CID: 5318517) into active site residues of ACZ97474. A: docked ligand in binding surface; B and C: ligand intraction with active site residues in neuraminidase using Chimera and Ligplot, respectively.

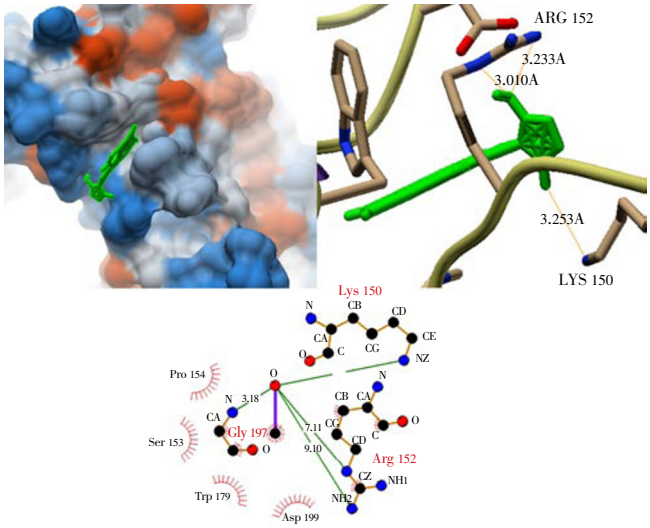

Figure 6. Dcoked Arabinoxylan (CID: 6438923) into active site residues of ACZ97474. A: docked ligand in binding surface; B and C: ligand intraction with active site residues in neuraminidase using Chimera and Ligplot, respectively.

Table 2

Characteristics of top ligand derivatives of 4-thiazolidinone identified form PubChem database after virtual screening, along with Food and Drug Admistraton approved drugs.

Compound name	LogP	M. W. (g/mol)	Binding energy (Kcal/mol)	No. of H-bonds	No. of hydrophobic interactions	Ki (nmol/L)	Total intermolecular energy (Kcal/mol)
Andrographolide (CID:5318517)	2.2	350.449	−10.88	3 (Arg152, Lys150, & Gly197)	4	10.59	−12.07
Arabinoxylan (CID:6438923)	−3.6	560.502	−6.64	5 (Arg118, Lys432, Ser367, Arg368, & Asn344)	2	13.48	−11.72
Epigallocatechin (CID:65064)	0.0	306.267	−7.93	2 (Ser367, Lys432)	4	1.54	−11.51
Zanamivir (CID:60855)	−3.2	332.309	−7.73	4 (Glu278, Glu228, Glu119, Trp179)	3	2.15	−10.12
Oseltamivir (CID:65028)	1.1	312.405	−6.28	2 (Lys432, Ser367)	9	156.39	−7.88
Imperatorin (CID:10212)	3.4	270.280	+50.37	2 (Ser367, Lys432)	9	–	+48.58
$C_{20}H_{30}O_5$ (CID: 29927575)	2.3	350.449	−10.90	3 (Arg152, Lys150, & Gly197)	3	10.27	−12.09
$C_{20}H_{30}O_5$ (CID: 6857767)	2.2	350.449	−10.90	3 (Arg152, Lys150 & Gly197)	4	10.26	−12.09
$C_{20}H_{30}O_5$ (CID: 11382524)	2.2	350.449	−10.90	3 (Arg152, Lys150 & Gly197)	3	10.22	−12.09
Andrographis $C_{20}H_{30}O_5$ (CID: 5318517)	2.2	350.449	−10.88	3 (Arg152, Lys150 & Gly197)	4	10.59	−12.07

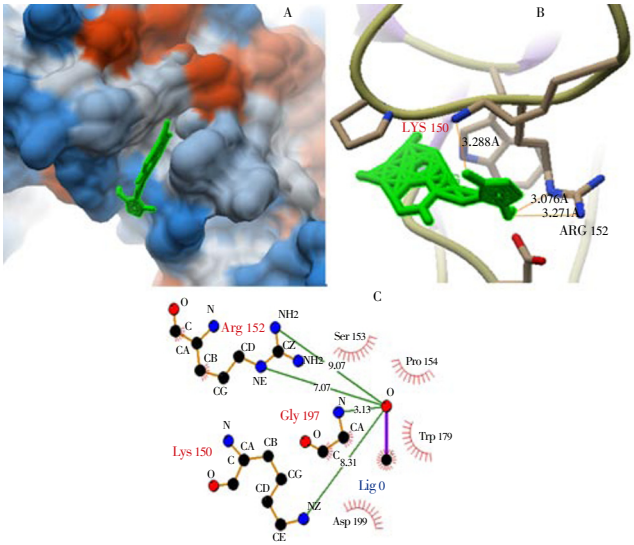

Figure 7. Dcoked Epigallocatechin (CID: 65064) into active site residues of ACZ97474.

A: docked ligand in binding surface; B and C: ligand intraction with active site residues in neuraminidase using Chimera and Ligplot, respectively.

Docking simulation of andrographolide derivatives to the homology modeled ACZ97474 was performed using Autodock4.2. All the 140 andrographolide ligands were docked into the defined binding site. The top 10 configurations after docking were taken into consideration to validate the result (Table 2). The RMSD value calculated out of ten accepted poses for each configuration was found in between 0.59-1.33 Å. This revealed that the docked configurations have similar binding positions and orientations within the binding site and are similar to the crystal structure. The best docked structures which are the configuration with the lowest binding score are compared with the crystal structure as shown in Figures 2-8. Docking of andrographolide derivatives to this binding site was performed using the standardized docking protocol. The binding mode of andrographolide within the binding site is represented in Figure 5. In this figure we can observe that both the molecules were well fitted to the defined binding pocket. All 140 andrographolide analogues were also found to be good binders with ACZ97474. The binding modes of andrographolide and its derivatives showed hydrophobic interaction with ACZ97474. This binding mode proved the hypothesis that the andrographolide derivatives bind to ACZ97474 with almost hydrophobic interactions and it should be the pre-organized shape binding between the rigid structure of andrographolide analogues and the binding pocket of ACZ97474. All the docking and interaction analysis were carried out as per our previous studies[32-33].

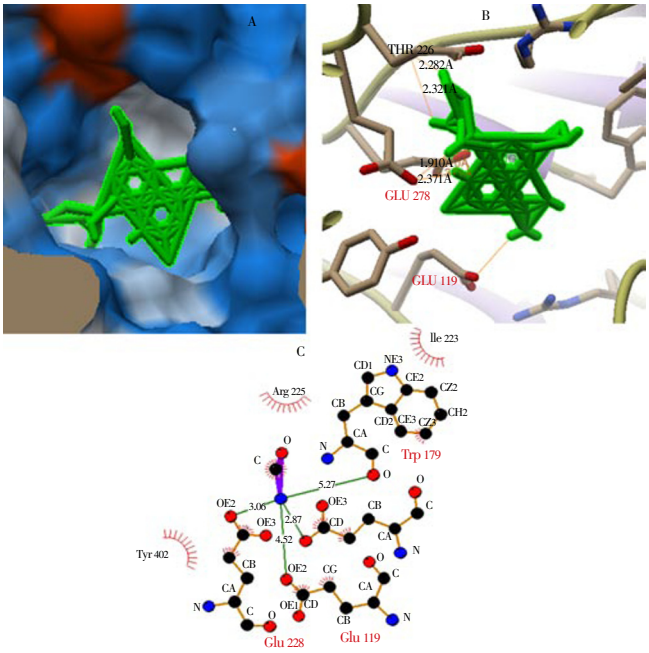

Figure 8. Dcoked Zanamivir (CID: 60855) into active site residues of ACZ97474.

A: docked ligand in binding surface; B and C: ligand intraction with active site residues in neuraminidase using Chimera and Ligplot, respectively.

4. Discussion

Highly pathogenic H1N1 influenza A viruses have spread relentlessly across the globe since 2003, more than 300 infections in humans have been reported, and the mortality rate is 60%[34]. Influenza A viral infection is still a major health concern, and the options for the

control and treatment of the disease are limited. In recent years the normally mortality associated with influenza H1N1 virus that circulated five years ago also hit younger people, is highly noticiable and this variant of the virus appears to be even more lethal. The strains of influenza H1N1 virus is unexpected mutate year to year, therefore it is important to get an annual flu vaccine[35].

Unfortunitely all vaccines are immune suppressive *i.e.* they suppress our immune system, which might increase the risk of contracting the flu or other infectious disease. Since vaccines bypass your natural first-line defense, therefore, they are never 100% protective and typically provides inferior immunity compared to our body would receive from naturally contracting and recovering from a disease. Additionally, Flu vaccinations are particularly ineffective which is reducing illness and mortality from the flu[36]. According to the US Centers for Disease Control and Prevention about 20% of flu-like illnesses are actually caused by influenza type A or B and the other 80% are caused by more than 200 other viruses such as respiratory virus, bocavirus, coronavirus, and rhinovirus, *etc*. Therefore this threat of a new pandemic requires the development of new therapeutic agents.

Docking results show that andrographolide (CID: 5318517) is the best among four herbal compounds as its carboxyl functional group binds with the active site residues Arg152 along with Lys150, and Gly197 of neuraminidase (ACZ97474) with lowest binding energy (-10.88 Kcal/mol) *i.e.* high binding effeciency, only 10 compounds including andrographolide show binding with Lys150 (150 loop) and active site Arg152 of neuraminidase. Total intermolecular energy was found to be -12.07 Kcal/mol with inhibition constant (Ki) of 10.59 nmol/L. These studies revealed structural conformation changes in 150 loop, secondary sialic acid binding site residues of neuraminidase. The guanidine group of Arg152 have binding affinities to the hydrophilic nature of the inhibitors (-OH and =O groups), as identified by docking, more interestingly, andrographolide was found to be most fitted ligand with ACZ97474 into active site residues than approved drugs for *Swine flu* such as Zanamivir (CID: 60855) and Oseltamivir (CID: 65028). Zanamivir was found interacting with Glu278, Glu228, Glu119, and Trp179 but have low binging affininty of -7.73 Kcal/mol compared to Andrographolide. Additionally, Oseltamivir also fails to fight with andrographolide in our study which proves that this information might be useful in designing future neuraminidase inhibitors for the rapidly mutating H1N1 strains.

Conflict of interest statement

We declare that we have no conflict of interest.

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