

Retinoid BMS411 (4-[(5,5-dimethyl-8-phenyl-5,6-dihydronaphthalen-2-yl) carbonyl] amino} benzoic acid), a potential inhibitor of NS5A protein of hepatitis C virus, a candidate for combined therapy of hepatitis C infection

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Summary. – Hepatitis C infection is a serious health issue worldwide caused by hepatitis C virus (HCV). There is an urgent need of search for new direct acting antiviral drugs due to the rapid development of drug resistance. The HCV NS5A protein is involved in creating resistance against antiviral therapy and there are also many reports that vitamin A deficiency is associated with non-responsiveness to antiviral treatment in HCV infected patients. So the present *in silico* study was aimed to find the relation between vitamin A deficiency and the NS5A protein's function in antiviral resistance. Structure of NS5A protein was predicted by using I-Tasser (Interactive Tasser). Previous data on conservative domains and dimer formation were confirmed by using a series of current computational methods. The structure was employed for molecular docking analysis to investigate the interaction of ligand BMS411 (4-[(5,5-dimethyl-8-phenyl-6H-naphthalene-2-carbonyl)amino]benzoic acid), a vitamin A related compound with NS5A protein. Docking analysis showed that retinoid BMS411 can bind to HCV NS5A protein and may act as inhibitor of this protein. The functionally interacting amino acid residues surrounding the ligand molecule were identified and were shown to be involved in the formation of binding pocket. The present study suggests that retinoids may play an important role in the improvement of the outcomes of antiviral therapy against HCV through interaction with NS5A and inhibition of this protein. It is of great importance to check and verify if other retinoids could act as NS5A inhibitors.

Keywords: hepatitis C virus; NS5A; docking; BMS411; retinoid; interferon therapy

Introduction

Hepatitis C is caused by a single-stranded RNA virus. Over 170 million people all over the world and 10 million people in Pakistan are affected by this virus (Raja and Janjua, 2008). HCV is a member of the *Flaviviridae* family of enveloped viruses, which infect nearly 2%–3% of the world's population with associated risk of end stage cirrhosis and hepatocellular

carcinoma in 80% of cases. Pakistan is experiencing burden of HCV infected population and high mortality rate. Hepatic malignancies including liver failure and hepatocellular carcinoma are the major cause of increased mortality rate in HCV infected individuals (Akbar *et al.*, 2009).

The genome of HCV contains 9600 nt in one open reading frame (ORF). A polyprotein of about 3,000 aa is encoded by the viral genome. Structural (core, E1 and E2) as well as non-structural (NS2, NS3, NS4A, NS4B, and NS5A/B) proteins are produced due to the posttranslational cleavage of poly-protein by the activity of viral and cellular enzymes (Reed and Rice, 2000). NS5A protein has achieved great attention among these structural and nonstructural proteins.

Hepatitis C virus is highly heterogeneous which is a serious obstacle in the progress of standard treatment and the

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Abbreviations: BMS411 = (4-[(5,5-dimethyl-8-phenyl-6H-naphthalene-2-carbonyl)amino]benzoic acid); HCV = hepatitis C virus; ISDR = interferon sensitivity determining region; NS5A = non-structural 5A protein; SVR = sustained virologic response

development of vaccine against HCV is therefore difficult. Viral genome is highly variable, so there are more than 50 subtypes of HCV and seven genotypes. About 70% of all infections in China, United States, Japan and Europe are caused by the subtypes 1, 1a and 1b (Kim and Saab, 2005). The currently approved HCV treatments are strongly determined by the HCV genotypes. It has become very difficult to develop a vaccine against hepatitis C viral infection due to high variability in genome sequence, so it is necessary to develop new therapies that have the ability to block different genotypes of HCV.

No effective vaccine is available for HCV infection and a success rate of 50% has been achieved by the use of interferon (IFN) along with nucleoside analogue ribavirin as standard treatment. Distinct regions of HCV polyprotein show amino acid variations that are responsible for phenotypic resistance to antiviral therapy. Several viral proteins involved in HCV lifecycle such as NS3, NS4A, NS5B RNA dependent RNA polymerase, and especially NS5A protein have been used as targets for developed of drugs. NS5A protein is a hydrophilic phosphoprotein composed of 447 aa. It is essential for the viral life cycle and may take part in genome replication, interferon response, cellular growth and apoptosis regulation. For its functions, it interacts with host cell proteins as well as other HCV proteins (Reed and Rice, 1997). The respective protein has anti-apoptotic potential and determines the cellular response to the interferon alpha. These functions have been marked over the past decade, revealing the vital role of NS5A for HCV (Macdonald and Harris, 2004). The identification of the interferon sensitivity determining region (ISDR) suggests that this protein is a major factor that determines the response of a patient to IFN- α . Several studies showed that in patients infected with HCV genotype 1a and genotype 1b, sensitivity to IFN- α is due to many mutations within ISDR of NS5A (Sarrazin *et al.*, 2000).

NS5A is a proline rich, three domains containing phosphoprotein. Domain 1 contains a zinc-binding motif and an amphipathic helix present at N terminal, which are essential for HCV replication and membrane association respectively (Peninet *et al.*, 2004). Within domain 1 four essential cysteine residues collectively bind to a zinc ion, and the complete inhibition of RNA replication may occur due to mutations of these residues (Tellinghuisen *et al.*, 2004). Domain 2 and 3 are unstructured and perform their functions by interacting with other viral and host cell proteins.

Recently in many countries, direct acting antiviral agents that are particularly targeted have become available for the treatment of HCV infection. However, they could not be used as an alternative to the combination therapy (interferon along with ribavirin) for many years because if used in monotherapy they are susceptible to favor the progress of resistance. Many factors have been recognized that can

help clinicians in the prediction of possibility of attaining sustained virologic response (SVR), a state with no detectable viral RNA in the blood 6 months after the completion of antiviral therapy, before starting the antiviral therapy for HCV infection (Asselah *et al.*, 2010). Among these factors, only some of them can be modified. Recently, levels of vitamin D and A in the serum appeared as new modifiable factors for the prediction of SVR rates. It has been shown that the effect of IFN I against the HCV replication can be increased through the up-regulation of the expression of IFN receptor by vitamin A (Hamamoto *et al.*, 2003). It has also been indicated that vitamin A exerts multiple effects in case of many diseases like respiratory and diarrhea diseases, measles infection, etc. In many under developed countries, deficiency of this vitamin affects the rates of mortality and morbidity (Haider and Bhutta, 2011).

In HCV infected patients that were treated with combination therapy (IFN along with ribavirin), deficiency of vitamin A was related to the lower SVR rates. These findings suggest that in order to increase the antiviral therapy outcomes, addition of vitamin supplements in the treatment could be helpful and vitamin A (retinoic acid) and its related compounds (retinoids) could be used as antiviral agents in future. BMS411 (4-[(5,5-dimethyl-8-phenyl-6H-naphthalene-2-carbonyl)amino]benzoic acid), a type 2 retinoid is used in this study as a ligand for NS5A protein for identification of active residue (functionally interacting amino acid residues) through docking analysis.

The critical role of NS5A in hepatitis C is undeniable. This protein is one of highly flexible proteins and its overall structure and functions are poorly understood due to the difficulties with experimental methods which are faced due to its flexibility. Such difficulties have diverted the attention towards the development of bioinformatics tools that are helpful in obtaining the information about the structure and functions of highly flexible proteins. The aims of present study are: the confirmation of the previous data on conservative domains and dimer formation of NS5A protein by using current computational methods; investigation of the interaction of ligand BMS411 with NS5A protein and identification of functionally interacting amino acid residues of NS5A protein through docking analysis.

Materials and Methods

Sequence retrieval and 3D structure prediction. The non-structural 5A (NS5A) protein sequence of HCV genotype 2a was retrieved from NCBI in FASTA format with Acc. No. ABH10008. Threading approach was used for structure prediction by using I-Tasser (Interactive Tasser). It selected the best model by using template 1Zh1A with 35% query coverage, 67% sequence identity and 1.30 normalized Z-score.

Structure validation of 3D model of NS5A. The selected 3D model of NS5A protein was further evaluated by using the evaluation tools like Rampage and ERRAT. Rampage created a Ramachandran (ϕ/ψ) plot and provided a simple view of the conformation of protein and ERRAT provided information about the quality of structures by the analysis of the statistics of non-bonded interactions between different atom types.

Assessment of protein interactions of NS5A. The protein interactions of NS5A were assessed by the STRING server. This server showed the closely associated partner of NS5A in *Homo sapiens*.

Analysis of domains/motifs and metal ion binding sites. InterProScan tool was used for protein sequence and functional analysis. This tool scanned the NS5A sequence against InterPro's signatures, which are predictive models provided by several different databases, and provided information about the domains and repeats in NS5A sequence. In addition to InterProScan, Motif Scan tool was also used for the prediction of domains/motifs present in the NS5A protein structure. In our predicted structure of NS5A, conserved amino acid residues involved in the formation of metal ion binding sites were located and visualized by using the PYMOL software. The involvement of conserved cysteine residues in the formation of Zn metal ion binding site was further confirmed through mutational analysis by using HOPE server.

Determination of receptor-ligand interaction. In order to understand the binding interaction of NS5A protein with its ligand, molecular docking approach was used. Ligand BMS411 of target protein i.e NS5A was retrieved from ZINC database with Acc. No. 01542874. For the determination of protein-ligand interaction, docking of predicted protein with the ligand BMS411 was done by using the docking software Autodock Vina. To run Autodock Vina,

MOL2 format of the ligand and pdf format of protein (receptor) were used as input files.

Identification of functionally interacting amino acid residues. Amino acid residues that were involved in the interactions between receptor protein and ligand molecule were identified during post-docking analysis through visualization tools. In addition to this, other servers/tools like DoGSiteScorer and active site predictor were also utilized for the prediction of amino acid residues that may be involved in the formation of active/binding site pockets and may play an important role in the receptor-ligand interactions and protein-protein interactions.

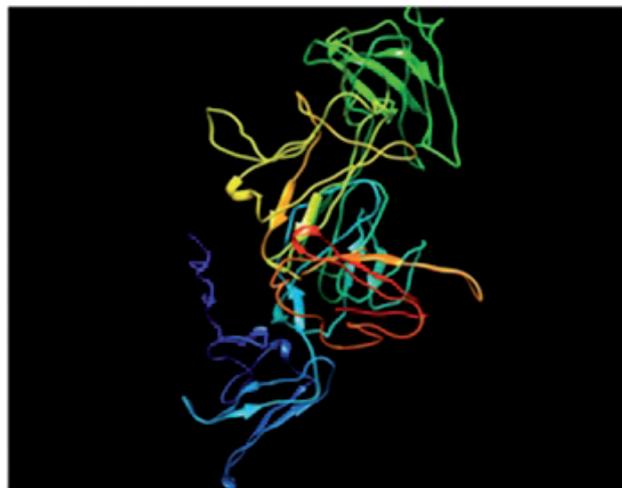


Fig. 1

I-Tasser predicted structure of NS5A

1Zh1A template with query coverage 35% was used for the prediction with I-Tasser.

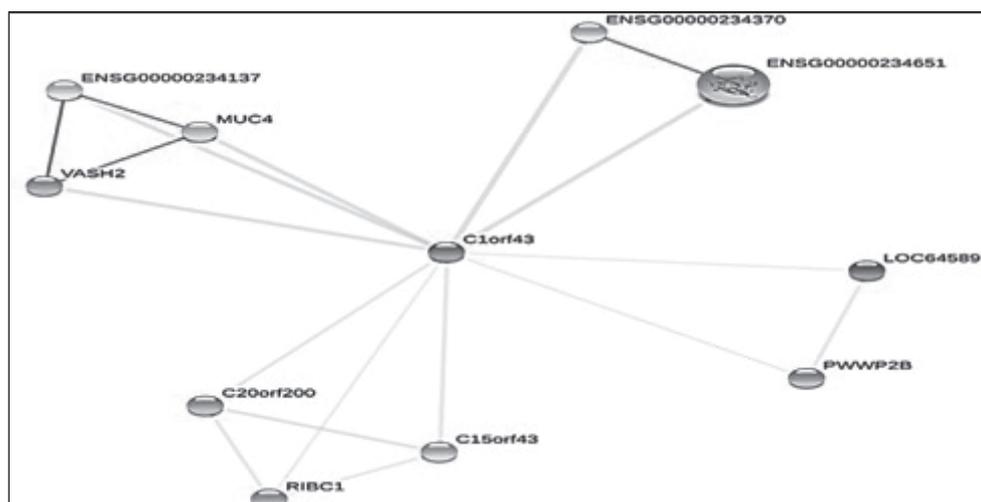


Fig. 2

Interaction network of C1orf43

Proteins that closely interact with protein C1orf43 (HCV NS5A-transactivated protein 4) a closely associated partner of NS5A in *Homo sapiens*.

Results and Discussion

This paper describes the implementation of an *in silico* technique to identify residues involved in binding of NS5A with its ligand. In many studies related to hepatitis C, the direct involvement of NS5A in producing disease has already been described. In current work we used an integrated bioinformatics approach to identify the active residues of candidate protein.

I-Tasser server predicted the top 5 models and selected the best model of NS5A by using the suitable template 1Zh1A with 35% query coverage, 67% identity and 1.30 values of normalized Z-score. The best model was selected on the basis of RMSD, TM score and C score. C-score is known as confidence score used for determining the quality of predicted models by I-Tasser. RMSD and TM score both are known standards which are used to measure the validity of structure modeling. TM score value higher than 0.5 shows that the predicted model has correct topology. The 1.52 value of C score and 0.53 value of TM score of our predicted model of NS5A proved correct topology of the model (Fig. 1).

The Rampage evaluation tool showed 68.1% favored region, 20.9% allowed region and 11% outlier region in NS5A. Backbone dihedral angles ψ against ϕ of amino acid residues in protein structure are visualized by the Ramachandran plot. Different regions are represented by the shading on the plot and the "core" regions representing the most favorable combinations of phi-psi values are represented by the darkest areas (Morris *et al.*, 1992). The evaluation tool Rampage showed that most of the psi/phi angle pairs for residues i.e. 68.1% were

Table 1. Domains/motifs in NS5A sequence predicted by Motif Scan tool

Positions	Motifs/Domains
292–439	Proline-rich region
2–24	Hepatitis C virus non-structural 5A protein membrane anchor
34–95	Hepatitis C virus non-structural 5A protein Zinc finger domain
96–196	Hepatitis C virus non-structural 5A protein domain 1b

present in favored region and very few residues were found in disallowed region showing that the predicted structure was of good quality. ERRAT demonstrated that the predicted model had a 65.438% quality factor (Peres *et al.*, 2011).

The protein interactions of NS5A were assessed by the STRING server. Uncharacterized protein C1orf43 (HCV NS5A-transactivated protein 4) was shown as closely associated partner of NS5A in *Homo sapiens*. Among several predicted functional partners of C1orf43, ENSG00000234370-general transcription factor 2H subunit 4 (general transcription factor 2H polypeptide 4; 462 aa) showed the closest interaction with score 0.899. Fig. 2 reveals the interaction network of C1orf43 showing the proteins that closely interact with this protein. Such close similarity was also observed by Pennin *et al.* (2004) in their *in silico* studies using similar computer based tools.

For protein sequence and functional analysis, InterProScan tool was used. InterProScan scanned the NS5A sequence against InterPro's signatures, which are predictive models

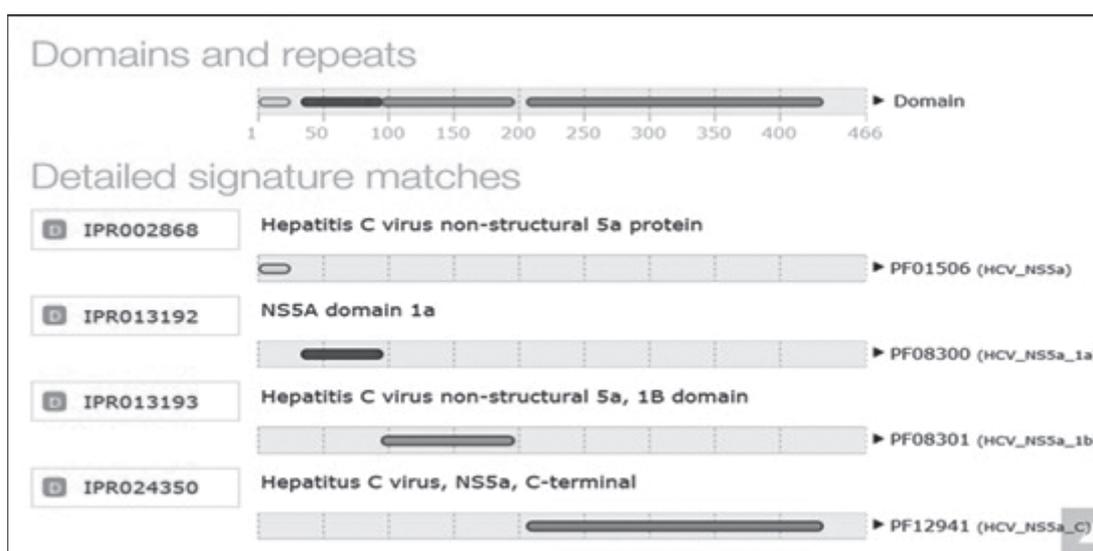


Fig. 3

InterProScan detailed signature matches of NS5A sequence

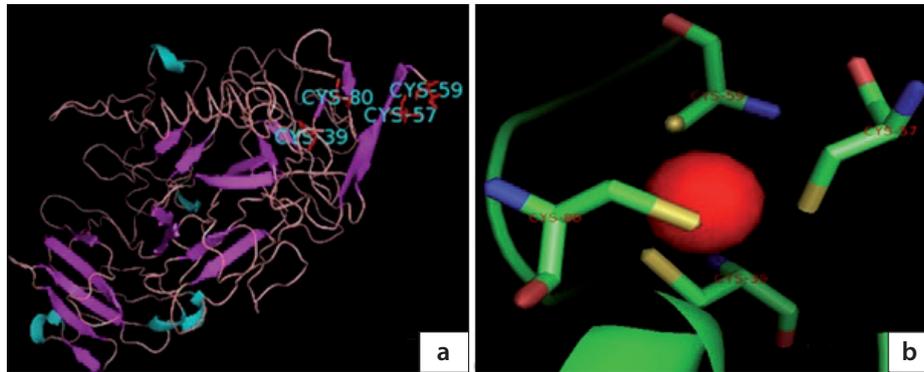


Fig. 4

I-Tasser predicted model for NS5A

(a) Model displays cysteine residues at positions 80, 59, 57, and 39 required for metal ion binding and NS5A dimerization. (b) Zn metal ion is represented by red colored sphere and four cysteine residues are shown surrounding the metal ion and forming binding site for Zn by their side chains.

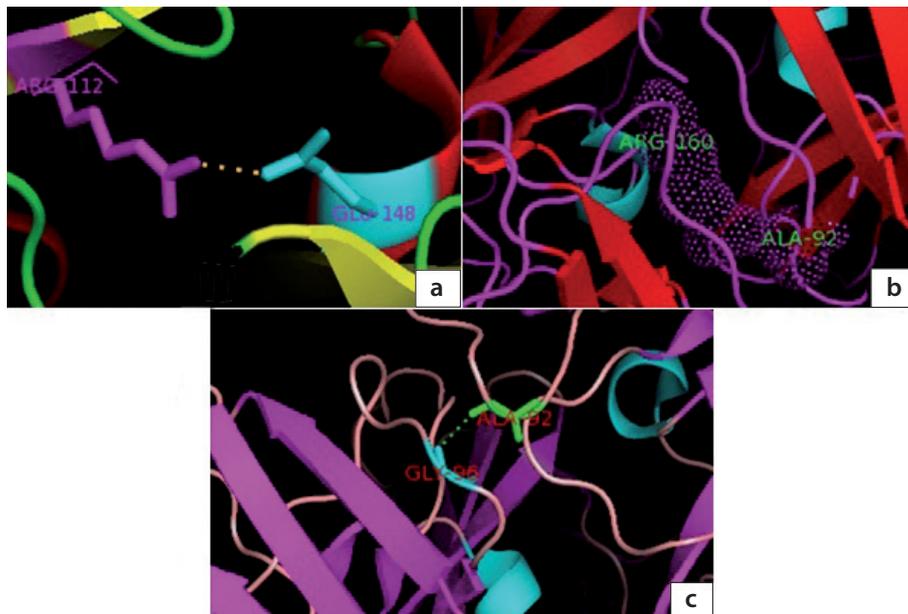


Fig. 5

Key intermolecular interactions shown by the main conserved chain atoms at the dimer interface of NS5A protein

(a) Highly conserved arginine 112 paired with glutamate 148 through intermolecular hydrogen bond; (b) arginine 160 interacting with alanine 92; (c) main chain atom alanine 92 interacting with glycine 96.

provided by several different databases. This tool analyzed the NS5A sequence and provided information about the domains and repeats of NS5A protein in addition with the detailed signature matches (Fig. 3). Motif Scan tool showed the presence of membrane anchor motif in the NS5A sequence at the positions 2–24. This tool also showed the strong matches of NS5A proline-rich region at position 292–439 and NS5A zinc finger domain at position 34–95. Domains/motifs in NS5A sequence predicted by Motif Scan tool are described in Table 1. Similar results have been also reported by Forester *et al.* (2009).

The NS5A domain 1 consists of a conserved motif known as zinc-binding motif and an amphipathic helix present at N-terminal that is involved in membrane association. Previously it has been shown that the N-terminal domain 1 of NS5A contains four cysteine residues at positions 80, 59, 57, and 39 that are involved in NS5A dimerization and are required for the function of NS5A in HCV replication (Tellinguisen *et al.*, 2004). Our selected model for NS5A also showed the conserved cysteine residues at positions 80, 59, 57, and 39 that serve as binding site for Zn metal ion

Table 2. Description of NS5A ligand retrieved from ZINC database

Receptor protein	Ligand database	Acc. No.	Ligand name	Formula
NS5A	ZINC	01542874	4-[[5,5-dimethyl-8-phenyl-5,6-dihydronaphthalen-2-yl]carbonyl]amino]benzoic acid	C ₂₆ H ₂₃ NO ₃

(Fig. 4). The domain 1 of NS5A protein exists as a dimer with main chain atoms and conserved residues involved in the stabilization of dimeric structure by forming intermolecular hydrogen bonds. Key intermolecular interactions at the dimer interface were visualized by PyMOL (Fig. 5).

AutoDock Vina docking software was used to investigate how the ligand binds to the respective protein, the binding conformation and functionally interacting residues. BMS411, a ligand of NS5A retrieved from ZINC database (Acc. No. 01542874) was used as ligand for docking study (Table 2, Fig. 6). Through different ligand conformations, nine complexes were created by the AutoDock Vina software and the complex having lowest docked binding energy

(-8.3) was selected for post-docking analysis using YASARA software and interactions between receptor and ligand were analyzed (Fig. 7). For the identification and visualization of residues involved in the formation of binding pocket at the active site of protein, PYMOL software was used (Asselah *et al.*, 2010). Figure 8 illustrates the protein-ligand post-docking analysis by PyMOL.

The amino acid residues GLN 378, SER 388, ALA 367, GLY 337, ARG 353, LEU 340, LEU 387, CYS 338, PRO 349, VAL322 and SER 324 in NS5A protein sequence were identified in the vicinity of ligand molecule. These amino acids were found to be involved in the formation of binding pocket for the ligand.

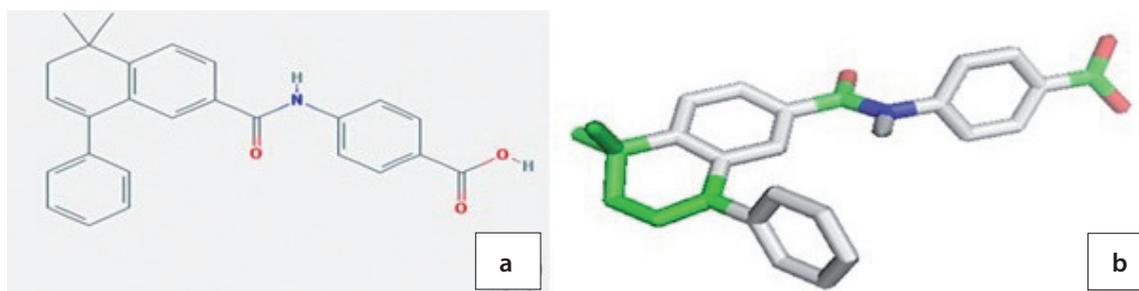


Fig. 6

Structure of BMS411

(a) Structure of NS5A ligand BMS411(4-[[5,5-dimethyl-8-phenyl-6H-naphthalene-2-carbonyl]amino]benzoic acid) used for receptor-ligand docking; (b) and its structure constructed by PyMOL.

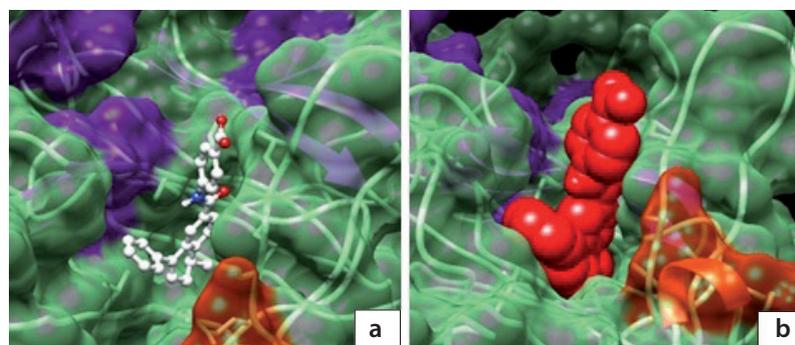


Fig. 7

Docked complex of NS5A with ligand BMS411 (frontal view)

(a) Receptor protein is represented in surface format, showing coils in green, strands in purple and helices in orange color. Ligand represented in ball and stick format is shown in white color; (b) ligand shown as red sphere.

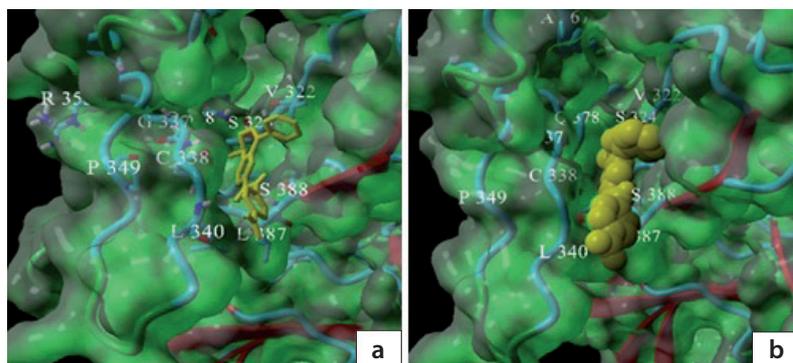


Fig. 8

NS5A binding pocket

NS5A docked complex with amino acid residues forming binding pocket visualized by YASARA. (a) Receptor protein is represented in surface format showing coils in blue and strands in red color and ligand BMS411 represented in stick format is in yellow color; (b) ligand is shown in sphere format.

In addition to this, an online server DoGSiteScorer was also utilized for the prediction of amino acid residues forming active/binding site pockets for NS5A protein ligands. The residues predicted by this server may be important for the determination of receptor-ligand interaction between NS5A and its inhibitors (Fig. 9). The best binding pocket forming amino acid residues predicted by the DoGSiteScorer server were almost same as identified by the docking studies.

In patients having chronic hepatitis C viral infection, deficiency of vitamin A was shown to be greatly associated with the stage of this disease i.e from mild conditions to chronic stage, and deficiency of this vitamin was observed at higher rates. Vitamin A deficiency in the serum and the non-responsiveness towards the antiviral agents used for the treatment of HCV infection are associated. Two reports have been issued regarding the association of vitamin A with HCV infection in chronic stage. The first report concerned drug users infected with both HCV and HIV infections (Forrester *et al.*, 2009) and the second report concerned patients infected with different phases of HCV infection (Peres *et al.*, 2011). All these findings suggest the strong association of vitamin A deficiency with non-responsiveness to antiviral treatment. By maintaining the serum vitamin A levels severity of HCV infection could be prevented.

In the present study, we selected the BMS411 a vitamin A related compound as a ligand for NS5A protein for docking analysis, because it has been predicted that this compound can bind with NS5A. A hypothetical model of NS5A binding with this compound has been proposed.

In order to find out whether this compound binds with NS5A protein, we performed docking analysis. Docking results showed the binding of HCV NS5A protein with BMS411 and revealed amino acid residues that are shown to be involved in the formation of binding pocket. Therefore we suggest that BMS411 can be used as potential inhibi-

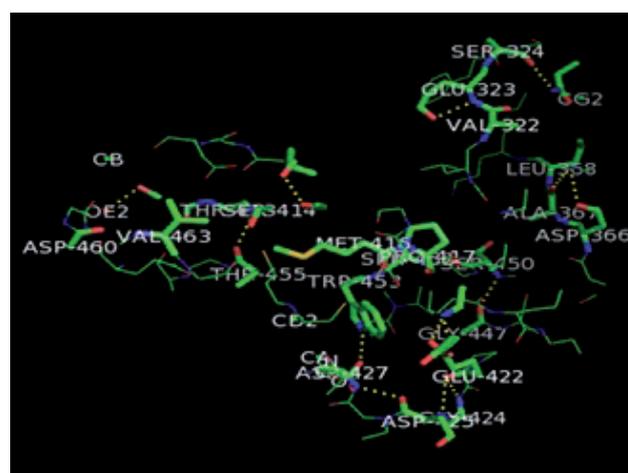


Fig. 9

The best binding pocket for NS5A ligands predicted by the DoGSiteScorer server

Amino acid residues forming the pocket visualized by PYMOL.

tor of HCV NS5A. Since the role of vitamin A deficiency has been proposed to be associated with the non-responsiveness to antiviral agents in the HCV infected patients, BMS411 a vitamin A related compound may be helpful in the reduction of non-responsiveness to antiviral therapy by inhibiting the NS5A protein. This finding suggests that retinoids may play an important role in the improvement of the outcomes of antiviral therapy against HCV. It would be of great importance to check and verify if other retinoids could act as NS5A inhibitors. This study also suggests that before antiviral treatment, normalization of serum vitamin A levels in hepatitis C patients and vitamin A supplementation can be helpful in the reduction of non-responsiveness to antiviral therapy.

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