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A comprehensive molecular interaction map for Hepatitis B virus and drug designing of a novel inhibitor for Hepatitis B X protein

Singh Jitendra[1], Ashina Nanda[2] *, Satveer Kaur[2], Maneet Singh[1]

[1]Department of Bioinformatics, ADI Biosolution, Mohali, Punjab, India-160059; [2]Department of Bioinformatics, Hans Raj Mahila Mahavidyalaya, Jalandhar, Punjab, India; Ashina Nanda – Email: nandasashina@yahoo.in; *Corresponding author

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Abstract:
Hepatitis B virus (HBV) infection is a leading source of liver diseases such as hepatitis, cirrhosis and hepatocellular carcinoma. In this study, we use computation methods in order to improve our understanding of the complex interactions that occur between molecules related to Hepatitis B virus (HBV). Due to the complexity of the disease and the numerous molecular players involved, we devised a method to construct a systemic network of interactions of the processes ongoing in patients affected by HBV. The network is based on high-throughput data, refined semi-automatically with carefully curated literature-based information. We find that some nodes in the network that prove to be topologically important, in particular HBx is also known to be important target protein used for the treatment of HBV. Therefore, HBx protein is the preferential choice for inhibition to stop the proteolytic processing. Hence, the 3D structure of HBx protein was downloaded from PDB. Ligands for the active site were designed using LIGBUILDER. The HBx protein's active site was explored to find out the critical interactions pattern for inhibitor binding using molecular docking methodology using AUTODOCK Vina. It should be noted that these predicted data should be validated using suitable assays for further consideration.

Keywords: Hepatitis B virus, HBx protein, PathVisio, Molecular-interaction map, Virtual screening, Docking, Inhibitor.

Background:
The World Health Organization (WHO) estimates that there are currently 400 million individuals worldwide who are chronically infected, of whom 100 million eventually will die **[1]** with the hepatitis B virus (HBV), it is critically important to understand how persistent HBV infection is maintained and linked to chronic hepatitis, cirrhosis, and development of liver cancer (hepatocellular carcinoma [HCC]) **[2]**. HBV is a member of the *Hepadnaviridae* family of viruses. The natural host for HBV is humans **[2]**, although similar viruses have been isolated from apes, woodchucks (woodchuck hepatitis virus [WHV]), squirrels (ground squirrel hepatitis virus [GSHV]), herons (heron hepatitis B virus), ducks (duck hepatitis B virus), geese (goose hepatitis B virus), and cranes (crane hepatitis B virus). The hepadnavirus genome is a partially double-stranded circular DNA structure that is encapsidated within the enveloped viral particle **[3]**. Upon infection of hepatocytes, viral DNA is transported to the nucleus, where it is converted to a covalently closed, circular, double-stranded DNA (cccDNA). The cccDNA is essentially an episome that does not replicate but functions solely as the template for all viral transcription **[3]**. The HBV genome is highly compact, such that over 50% of the coding capacity lies in at least two open reading frames. Genomic and subgenomic viral transcripts, all of which utilize the same polyadenylation signal, are all transcribed from cccDNA. Viral mRNAs encode the viral core (HBcAg), envelope (HBsAgs), polymerase/reverse-transcriptase (pol), and HBx polypeptides. The largest HBV transcript, the pregenomic RNA (pgRNA), is also the template for viral replication and is reverse transcribed by the viral pol, similar to the replication of

retroviruses **[4]**. However, in contrast to that of conventional retroviruses, reversetranscribed HBV DNA is not integrated into the host cell genome as a requisite step in the viral life cycle. Instead, an intermediate in the replication reaction, a partially double-stranded viral DNA genome, is encapsidated within the mature viral particle. Viral pgRNA is encapsidated in cytoplasmic viral particles comprised of core protein, known as core particles, within which reverse transcription and DNA replication occur. As the virus replicates, it buds into the endoplasmic reticulum by envelopment within the viral HBsAg proteins and is eventually secreted from the infected cell **[2]**. While all hepadnaviruses can establish persistent infections in their respective hosts, only chronic infection by mammalian hepadnaviruses is associated with significant development of HCC **[2]**. As avian viruses either lack the HBx gene or, at best, encode a highly divergent form, the potential role of HBx in the development of HCC in mammals has been an area of intense interest for research **[5]**.

Role of hepatitis B virus proteins:

In addition to the integration of viral genome into host DNA, another direct role of HBV in hepatocarcinogenesis involves the long-term expression of viral proteins such as X protein (HBx) **[2, 6]**. This is supported by the observation that in a large portion of HCCs, viral DNA sequences encoding these proteins are found to be integrated in the host genome **[8]**. Hepatitis B virus has a unique fourth ORF coding for a protein known as HBx **[9-11]**. The HBX gene is well conserved among the mammalian hepadnaviruses and codes for a 16.5 kDa protein, which has been detected in both the nucleus and the cytoplasm. HBX mRNA (0.7 kb) has been detected in infected liver, but the protein has not been easy to detect. HBX gene has been shown to be essential for the establishment of HBV infection in vivo **[7]**. HBx likely binds directly to the transcription factor CREB and possibly to transcription factors TFIIB, TFIIH, and the RNA polymerase II-associated protein RPB5, but the latter might interact with HBx via secondary interactions with CREB. HBx-CREB transcription complexes have been shown to stimulate transcription. HBx is thought to act on the mitochondria, causing calcium release, in turn activating the Pyk2/FAK and Src kinase families, leading to stimulation of a variety of cytoplasmic signal transduction pathways. Interaction of HBx with the UV-DDB proteins (1 and/or 2) is implicated in stimulation of viral replication. HBx interaction with the proteasome is also reported to stimulate HBV replication **[2]**.

HBx is a promiscuous transactivator, it activates a variety of viral and cellular promoters in diverse cell types, and it has been shown to transactivate several viral and cellular targets. It can activate p53 but can also influence a variety of signal transduction pathways like MAPK/ERK, protein kinase B (PKB), PKC, SAPK/JNK, Phosphatidylinositol 3-Kinase (PI-3-K) and janus kinase (JAK)/STAT kinase pathways within the cell. HBx up regulates the activity of a number of transcription factors. However, the majority of HBx is localized to the cytoplasm where it interacts with and stimulates protein kinases. It can also localize to the mitochondrion where it acts as an adaptor or kinase activator to influence signal transduction pathways **[7, 12]**. HBx can bind to C-terminus of p53 forming a proteinprotein complex and inactivating many functions of p53, including apoptosis. HBx sequester p53 in the cytoplasm and prevents it from entering the nucleus. HBV protects itself from apoptotic death through an HBx-P13K-AKT-Bad pathway and by inactivating caspase 3 activity that is partially p53 independent in hepatocytes.The proapoptotic action of HBx overcomes the inhibitory effect of Bcl-2 against Fas cytotoxicity. HBx also up regulates surviving expression, an inhibitor of apoptosis (IAP) family. HBx also promotes the apoptosis of liver cells by upregulating the expressions of Fas/Fasl, Bax/Bcl-2, Bcl-xL, and c-myc in a dose dependant manner. Thus HBx has function in both pro-apoptosis and anti apoptosis pathways dependent on different cell settings. Increased anti-apoptosis and decreased pro-apoptosis in HCC is an important mechanism **[13]**. The integrated HBx is frequently mutated and has a diminished ability to function as a transcriptional cotransactivator and to activate the nuclear factor kappa-B (NF-κB) pathway **[7]**.

In this study we present the first step towards an integrated construction of Molecular-Interaction Network pathways in Hepatitis B virus. Our focus is on the interactions of the protein HBx, as this protein HBx play a central role in regulation of hepatitis, cirrhosis, and development of hepatocellular carcinoma. This HBx protein is involved in the cell cycle regulation, apoptosis, signalling, transcriptional regulation, encoding of cytoskeleton, cell adhesion molecules, oncogenes and tumour suppressor genes. Therefore, understanding the integrated function of this protein is of great importance in development of effective therapies for these diseases. In the second steps of this study we designed an inhibitor which showed inhibitory activity towards Hepatitis B virus HBx protein. The binding interactions between this inhibitor and HBx protein were studied by docking methods using AutoDock vina software. The aim of this study was to get a better ligand that could inhibit polyprotein processing of HBV, and to better understand the interactions between the inhibitor and the enzyme's binding sites via computational docking methods. We hope, this Drug will get success to clear out all the phases of clinical trial and it will be effective drug in the cure HBV's diseases.

Methodology:

Data:

Using the search terms 'Hepatitis B virus AND hepatitis b virus disease AND hepatitis b virus x protein', an intensive literature search of scientific papers was done in order to identify genes, proteins and small molecules that relate to HBV. Finally, peer-reviewed articles had been enrolled and combined with data publicly available in the KEGG database 'http://www.genome.jp/kegg/' in order to further identify proteins involved in HBV and to aid reconstruction of pathways.

Construction of the Molecular-Interaction Network:

Using the data obtained, the general molecular interaction network was created in PathVisio v2.0.11. Initially, connections were built among all the molecules (proteins, genes, DNAs, simple molecules, ions and antisense RNA) presented in the literature studied. In some cases, detailed regulatory relationships between different molecules, such as activation, inhibition and phosphorylation were available, enabling the re-construction of part of the HBV map. In the case where

molecules were identified in the literature, but their interactions not identified, we searched the KEGG PATHWAY database for missing connections. Where no interaction information was available from either the literature or KEGG database, the molecules were excluded from the map. Each entity in the network is annotated with a complete list of interactions in which it participates, and PMIDs supporting those interactions (**Figure 1**). Entities are also linked to Uniprot, NCBI and KEGG pages. From this Molecular-Interaction Network of HBV, HBx protein was taken for further in silico structure based drug designing study.

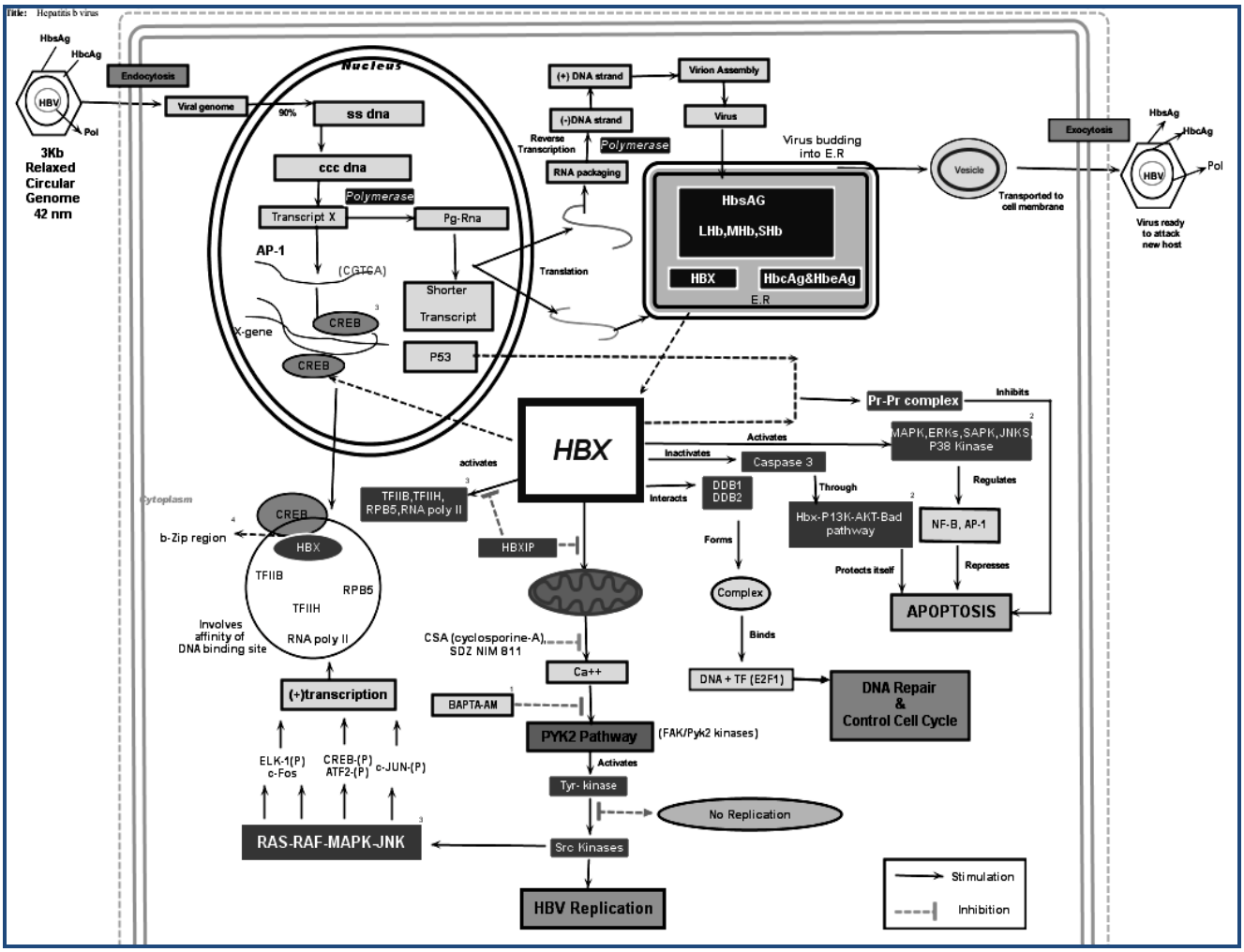

Figure 1: Molecular-interaction map for Hepatitis B virus.

HBx as a Target Protein:

Out of all the entries for HBx protein from RCSB protein data bank, HBx protein 3I7H was taken for docking analysis. The active site residues were found to be 88- ILPKVLHKRTLGLS -101.

Generating novel ligands:

The structure of the fragment, i.e. the "seed molecule" was revealed on the basis of previous studies of available inhibitors for HBx protein **[1]**. The fragment "(2S, 3R)-2-(hydroxymethyl) tetrahydrofuran-3-ol" was identified on the basis of "Lipinski's Rule of Five" and may therefore represent suitable starting point for evolution of good quality lead compounds. The docking analysis of "(2S, 3R)-2-(hydroxymethyl) tetrahydrofuran-3-ol" compound with HBx protein was carried out by HEX (http://hex.loria.fr/) docking software with default parameters. The conformation of the pre-placed "seed" ensuring the binding affinity decides the manner that ligands would be grown with Ligbuilder software. Novel ligands had been developed with Ligbuilder (http://mdl.ipc.pku.edu.cn/drug_design/work/ligbuilder.html) v1.2 software. We developed 100 novel ligands for the inhibitory site in HBx protein.

Virtual screening:

Virtual screening, an in silico tool for drug discovery, has been widely used for lead identification in drug discovery programs. Out of 100 novel ligands generated, 10 ligands were selected on the basis of maximum binding affinity measured in kcal/mol. The selected 10 ligands were then analyzed for drug- relevant properties based on "Lipinski's rule of five" and other drug like properties of valid structures using OSIRIS Property Explorer

(http://www.organic-chemistry.org/prog/peo/), Molsoft: Drug- Likeness and molecular property explorer (http://www.molsoft.com/mprop/). On the basis of binding affinity and drug like properties, one ligand that passed all of the screening tests was taken for further molecular docking study.

Protein-ligand docking:
The docking of ligands to the catalytic triad of HBx protein for HBV was performed using AutoDock Vina software. Docking was performed to obtain a population of possible conformations and orientations for the ligand at the binding site. Using the software, polar hydrogen atoms were added to the HBx protein and its nonpolar hydrogen atoms were merged. All bonds of ligands were set to be rotatable. All calculations for protein-fixed ligand-flexible docking were done using the Lamarckian Genetic Algorithm (LGA) method. The grid box with a dimension of 40 x 40 x 40 points was used around the catalytic triad to cover the entire enzyme binding site and accommodate ligands to move freely. The best conformation was chosen with the lowest docked energy, after the docking search was completed. The interactions of complex HBx protein-ligand conformations, including hydrogen bonds and the bond lengths were analyzed using Accelrys DS Visualizer software (http://accelrys.com).

Molecular descriptors calculation:
Quantitative structure-activity relationships (QSARs) correlate the response with molecular properties of compounds under interest. Any compound to be considered as a lead must possess acceptable scores for all of the descriptors. The OSIRIS Property Explorer (http://www.organic-chemistry.org/prog/peo/), Molsoft: Drug-Likeness and molecular property explorer (http://www.molsoft.com/mprop/), and Molinspiration property explorer (http://www.molinspiration.com/cgibin/properties/) were used to calculate 18 descriptors- logP, solubility, drug likeliness, polar surface area, molecular weight, number of atoms, number of rotatable bonds, volume, drug score and number of violations to violations to assure its drug-confirm behavior.

Results and Discussion:
Construction of the Molecular-Interaction Network:
We find that some nodes in the network that prove to be topologically important, in particular HBx, Polymerase, HBsAg and HBcAg & HBeAg, are also known to be associated with drugs used for the treatment of Hepatitis B. Importantly, based on topological consideration, we are also able to suggest HBx as a novel potentially relevant target protein for the diagnosis or treatment of Hepatitis B. This type of finding proves the potential of in silico analyses able to produce highly refined hypotheses, based on vast experimental data, to be tested further and more efficiently. As research on Hepatitis B is ongoing, the present map is in fieri, despite being -at the moment- a reflection of the state of the art.

HBx as a Target Protein:
Via the pathway analysis, we also found that HBx is associated with a number of pathways, for instance, the cell cycle regulation, apoptosis, signalling, transcriptional regulation, encoding of cytoskeleton, cell adhesion molecules, oncogenes and tumour suppressor genes. So it is reasonable here to suggest that HBx could be an interesting candidate as potential new drug target for the treatment of Hepatitis B. This is consistent with the recommendation in **[13].**

Protein-ligand docking:
The Protein-ligand interaction plays a significant role in structure based drug designing. Overall, the best confirmation showed that the free energy of binding (ΔGbind kacl/mol) for the designed ligand were good and represented in **Table 2 and Figure 2A & B**. The negative and low value of ΔGbind indicates strong favorable bonds between HBx protein and the novel ligand indicating that the ligand was in its most favourable conformations. The information about the number of hydrogen bonds formed and catalytic site residues involved in protein-ligand complex are shown in Table and figure.

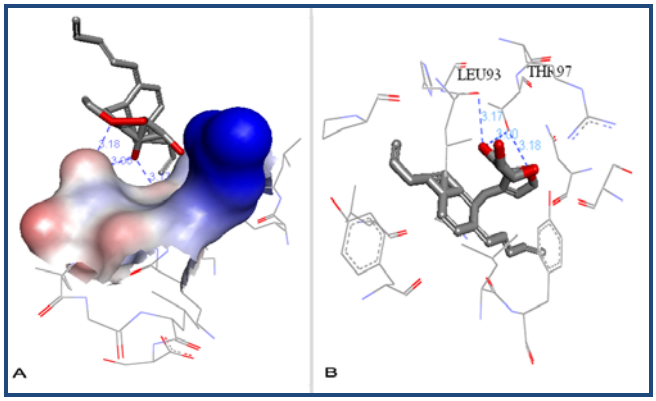

Figure 2: Docking representations of HBX protein. **(A)** Docked Ligand inside the pocket of HBX protein. **(B)** H-Bond interactions (blue dashed line) of the docked ligand with Active site residues.

Molecular descriptors calculation:
The further descriptor analysis and the toxicity prediction **(Tables 1 see supplementary material)** helped in the identification of the better inhibitor. Drug Score and the Drug-Likeness are the two properties that are important for considering a compound to become a successful drug. Novel ligand had a drug score of 0.29 and drug likeness property score of 0.49. The molecular weight of novel ligand was 398.619 g/mol between the preferred ranges of molecular weight for drug likeness property. From **Table 1** novel ligand was the compound that had the acceptable range (green colors) for toxicity risk. These values were also taken into account to decide the best inhibitor. Thus, novel ligand was the best drug candidate and also found to possess better global binding affinity score.

Conclusion:
In this study we have successfully reconstructed and analysed a systemic network of interactions of the processes on-going in patients affected by Hepatitis B. Through the topological analysis of this Hepatitis B network, HBX has been identified to be a hub of pathway. In this study, we designed a novel ligand against HBX protein of HBV. The molecular docking was applied to explore the binding mechanism and studies on the novel ligand against the HBX protein showed that the free binding energy for the inhibitor was small, indicating that the

ligand binds favorably to the binding site. The ligand was observed as the best inhibitor candidate, which may be considered as a potential ligand for treatment of diseases caused by HBV. Using a combination of virtual screening, and molecular docking, we successfully identified putative novel inhibitor, which can be further evaluated by in vitro and in vivo biological tests.

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Supplementary material:

Table 1: Molecular descriptors value for novel inhibitor.

Descriptors	Lead compound Scores
LogP	4.65
Solubility	-4.48
Molecular weight	398.619
TPSA	69.918
n Atoms	28
n Violations	1
N Rotatable bonds	12
Volume	426.017
Number of stereo centers	5
Number of HBA	4
Number of HBD	3
Mutagenic	Green
Tumorigenic	Green
Irritant	Green
Reproductive Effective	Green
Drug likeness	0.49
Drug score	0.29
Molecular formula	C24H46O4

Table 2: The number of Hydrogen bonds formed and the catalytic site residues involved in protein-ligand complex along with binding affinity.

Virus protein	**No. of Hydrogen bonds**	**Catalytic site residue(s) in Hydrogen bonding**	**Binding Affinity (Kcal/mol)**
HBX	1,2	Leu93,Thr97	-7.2