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Analysis of hepatitis B virus drug-resistant mutation (M204V) using molecular dynamics simulation techniques

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Abstract: Hepatitis B virus (HBV) is the most common cause of liver infection. This can be treated by targeting HBV DNA polymerase (HDP). Although many drugs are available for the treatment, lamivudine is widely used because of its good safety profiles. Lamivudine targets the reverse transcriptase activity of HDP and restricts the viral growth. However, the available literature evidence showed that the mutations in the catalytic site of tyrosine (Y203), methionine (M204), aspartic acid (D205) and aspartic acid (D206) will significantly reduce the efficacy of lamivudine and creates a resistance. In particular, M204V mutation affects the drug binding mechanism and cause resistance to lamivudine. Therefore, in the present study, we made an attempt to understand the mechanism of lamivudine resistance with the aid of computational techniques. The docking results suggest that lamivudine was found to adopt most promising conformations with native type HDP by identifying M204 as a prospective partner for making polar contacts as compared to the mutant type HDP. The molecular dynamic (MD) results showed that the average atom, especially atoms of the native-type HDP-lamivudine complex, movements were small, displayed fast convergence of energy and charges in geometry. This highlights the stable binding of the lamivudine with native-type HDP as compared to mutant type HDP. The normalized mean square displacement and root mean square fluctuation analysis certainly indicates conformational changes in the HDP structure due to M204V mutation. Furthermore, the hydrogen bond analysis from the MD study demonstrated that there is decreased number of intermolecular hydrogen bonds in mutant HDP-lamivudine complex than that in the native-type HDP-lamivudine complex.

Key words: HBV DNA polymerase; drug-resistance; molecular docking; molecular dynamics simulation.

Abbreviations: HBV, hepatitis B virus; HDP, hepatitis B virus DNA polymerase; MD, molecular dynamics; PDB, Protein Data Bank; $<R^2>$, normalized mean square displacement; RMSD, root mean square deviation; RMSF, root mean square fluctuation; RT, reverse transcriptase; SMILES, simplified molecular-input line-entry system; YMDD, tyrosine, methionine, aspartate, aspartate.

Introduction

Hepatitis B virus (HBV) infection is a global public health care problem and it is a common cause of liver diseases including liver cancer. HBV infection is seen in about two billion people, in which 350 million of them are chronic carriers (Lunel-Fabiani et al. 2013). HBV is highly infectious and transmitted mainly through blood, body-fluid contact, and vertical transmission (Baha et al. 2013). HBV belongs to the Hepadnaviridae family (Wynne et al. 1999) and it is a small DNA virus that replicates through reverse transcription (Pollack & Ganem 1994). It can be classified into six genotypic families: A, B, C, D, E and F. Most importantly A and D account for 90% of cases of HBV infection (Mayerat et al. 1999). Based on the antigenic properties, HBV expresses four major viral proteins: precore/core, surface, polymerase and HBV X protein (HBx), of which viral polymerase plays a key role for genome replication (Kim et al. 2010). The virally encoded reverse transcriptase (RT) contains initiation and elongation activity, helps in the reverse transcription of the double-stranded DNA genome of hepatitis B from the viral pregenome (Seifer & Standring 1993). Since the HBV RT plays an important role in the viral life cycle, antiviral drugs are developed to target the RT activity of the HDP (Lin & Hu 2008).

The ultimate aim of therapy for hepatitis B is to prevent the progression of the disease and to prolong the survival of the patient (Zoulim 2011). The antiviral drugs, such as lamivudine, telbivudine, entecavir, adefovir and tenofovir are currently used to treat HBV infection (Seifer et al. 2009). Lamivudine, the (-)enantiomer of the deoxycytidine analogue 2'-

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deoxy-3'-thiacytidine, rapidly suppresses HBV replication, and it proved to have an excellent safety profile. Hence, it has been widely used for the treatment against HBV infections (Dikic et al. 2001; Chang et al. 2006). Basically, this inhibitor is an oral nucleoside analogue that inhibits HBV DNA synthesis by chain termination during the replication cycle of HBV (Dikic et al. 2001).

However, the major limitation in the lamivudine treatment is the development of drug-resistant HBV after few months of antiviral therapy. The cause of resistance is due to the mutation of the catalytic domain of the HBV polymerase gene (Yuen & Lai 2003) and the element for primary resistance to lamivudine was mapped to mutations in the tyrosine, methionine, aspartate, aspartate (YMDD) motif (Kim et al. 2010). The YMDD motif is a highly conserved segment of the amino acid sequence involved in the deoxynucleoside triphosphate binding in the catalytic site of HDP. The amino acid changes in the HDP that affects lamivudine sensitivity involve the methionine at position 204 (M204) within the YMDD motif (Gaillard et al. 2002). Particularly, the M204V mutation in the YMDD catalytic motif in the C domain significantly alters the binding affinity of lamivudine and confers to drug resistance (Seifer et al. 2009).

Hence, the present study has focused on understanding the structural characteristics behind the M204V mutation with the aid of molecular docking and molecular dynamics (MD) approaches. Furthermore, drug-target interactions were also extensively analyzed in terms of prevalence of intermolecular hydrogen bonds interactions. We hope that these results will be helpful in understanding drug-resistance mechanism of the M204V mutation as well as will be useful for designing new lead compounds with potential therapeutic effects against the HBV infection.

Material and methods

Data set

The structure of native type of HDP was modelled using the SWISS-MODEL (Schwede et al. 2003) and the mutation M204V was generated using Swiss-PDB viewer (Guex & Peitsch 1997). Subsequently 1,000 steps of energy minimization were carried out for both native and mutant structures using GROMACS package 4.5.3 (Spoel et al. 2005; Hess et al. 2008). Lamivudine was used as the small molecule/inhibitor for our investigation. The SMILES strings were collected from PubChem, a database maintained in the National Center for Biotechnology Information (Feldman et al. 2006), and submitted to CORINA for constructing the three-dimensional structure of lamivudine (Gasteiger et al. 1990).

Homology modelling and flexibility of binding residue by normal mode analysis

A homology model of HDP was obtained using previously described methods (Langley et al. 2007; Daga et al. 2010; Ismail et al. 2010; Kim et al. 2010). HIV-1 RT shares a sufficient structural and functional similarity with HDP and can serve as a template for modelling the tertiary structure of HDP (Daga et al. 2010). The model for the native HDP was thus constructed with SWISS-MODEL (Schwede et al. 2003) using the structure of the HIV-1 RT [Huang et al. 1998; Protein Data Bank (PDB) code: 1RTD] as the template structure. The modelled protein was energy-minimized using the GROMACS package 4.5.3 (Spoel et al. 2005; Hess et al. 2008). Subsequently, the modelled structure was validated by Ramachandran plot using the PROCHECK server (Laskowski et al. 1993).

The atomic motions in proteins can be quantitatively obtained from the mean square fluctuations of the atoms corresponding to their average positions (Hirs et al. 1986). These can be related to the normalized mean square displacements ($<R^2>$) (Ringe & Petsko 1986; Yuan et al. 2005). Therefore, <R2> analysis is likely to provide newer insights into protein dynamics, flexibility of amino acids, and protein stability (Parthasarathy & Murthy et al. 2000). It is worth mentioning that protein flexibility is important for protein function, rational drug design, and maintaining various types of interactions (Carlson & McCammon 2000). Moreover, flexibility of amino acids in drug binding pocket is considered to be a significant parameter to understand the binding efficiency. In fact, the loss of flexibility in the binding site residues results in the decrease in binding free energies of the protein-drug complex (Hinkle & Tobacman 2003; Rajasekaran et al. 2008; Karthick et al. 2012, 2013). Hence, we computed the <R2> values with the aid of Elnemo program (Suhre & Sanejouand 2004) in order to understand the flexibility of the binding residues both in the native and the mutant types.

Computation of docking energy

To investigate the free energy of binding between the ligand and the target protein, docking simulation was performed using AutoDock 4.2. The software performs docking simulation based upon Lamarckian genetic algorithm method (Morris et al. 1998). The target protein was kept rigid throughout the docking simulation. In AutoDock, the grid-based approach was used to approximate the energy calculations used by the energy function. Grid box with 60 Å grid size (x, y, z) = (33.155, 54.290, 26.310) was created with a grid spacing of 0.375 Å (Fig. 1). Polar hydrogens, salvation parameters and Kollman charges were added into the receptor PDB file for the preparation of protein in docking simulation, while Gasteiger charges were added on the ligand PDB file. AutoDock tools were used to prepare, run and analyze the docking simulations (Morris et al. 2009). Finally, LIGPLOT (Wallace et al., 1995) was used to plot the intermolecular interactions between HDP and lamivudine.

Molecular dynamics simulation

The docked complex structures, such as native-type HDP-lamivudine and of mutant-type HDP-lamivudine were used as starting point for MD simulation. The GROMACS package 4.5.3 (Spoel et al. 2005; Hess et al. 2008) adopting the GROMOS43a1 force field parameter was used to perform the MD simulation. The structures were solvated in boxes of linear size of 6 nm, using the periodic boundary conditions and the SPC water model (Meagher & Carlson 2005). PRODRG server (Schuttelkopf & Van Aalten 2004) was used to generate ligand topology. One thousand steps of steepest descent energy minimization were carried out for the proteins. After energy minimization, the system was equilibrated at constant temperature and pressure. The system is equilibrated by performing the position-restrained dynamics simulation (NVT and NPT) at 300 K at time period of 300 ps. The equilibrated structures were then subjected to MD simulations for 15,000 ps with the integration time step

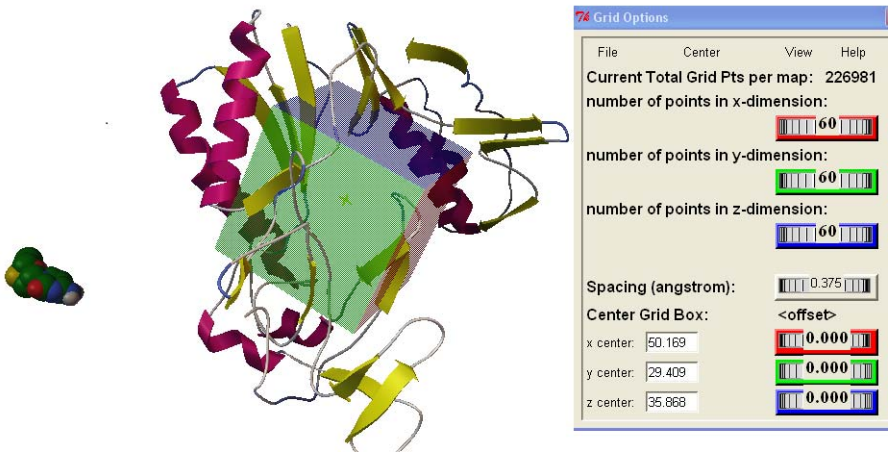

Fig. 1. AutoDock Grid box with 60 Å grid size (x, y, z) = (50.169, 29.409, 35.468).

```
HDP             ------CSDYCLTHIVN--LLEDWGPCTEHGEHNIRIPRTPARVTGGVFLVDKNPHNT-- 50
HIV_RT          PISPIETVPVKLKPGMDGPKVKQWPLTEEKIKALVEICTEMEKEGKISKIGPENPYNTPV 60
                          *.  ::   :::*    *: :  :.*     :      :  :**:**

HDP             --------TESRLVVDFSQFSRGSIHVSWPKFAVPNLQSLTN-----LLSSNLTWLSLDV 97
HIV_RT          FAIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAYFSVPL 120
                       *: * :*** ::.: : ..   ::.:*:  .*.:     :*. . :::*: :

HDP             SAAF-----YHIPLHPAAMPHLLVGSSGLPR-----------YVARLSSTSRNINYQHGT 141
HIV_RT          DEDFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRKQNPDIVI 180
                .  *     : **      * :    . **:            :::: .. *: * :

HDP             MQDLHDSCS-------------RNLYVSLLLLYKTFGRKLHLYSHP-IILGFRKIPMGVG 187
HIV_RT          YQYMDDLYVGSDLEIGQHRTKIEELRQHLLRWGLTTPDKKHQKEPPFLWMGYELHPDKWT 240
                 * :.*               .:*   **    *   * *  . * : :*:.  *

HDP             LSPFLLAQFT-------------------------SAICSVVR--RAFPHCLAFSYMDD 219
HIV_RT          VQPIVLPEKDSWTVNDIQKLVGKLNWASQIYPGIKVRQLCKLLRGTKALTEVIPLTEEAE 300
                :.*::*.:                              :*.::*  :*:.. :.::   :

HDP             VVLGAKSVQHLESLFTSITNFLLSLGIHLNPNKTKRWGYSLN---FMGYVIGSWGTLP-- 274
HIV_RT          LELAENREILKEPVHGVYYDPSKDLIAEIQKQGQGQWTYQIYQEPFKNLKTGKYARMRGA 360
                : *. :     *.:.    :   .*  .:: :   :* *.:    * .   *.:. :

HDP             QEHIVLKLKQCFRKLPVNRPIDWKVCQRIVGLLG---FAAPFTQCGYPALMPLYACIQAK 331
HIV_RT          HTNDVKQLTEAVQKITTESIVIWGKTPKFKLPIQKETWETWWTEYWQATWIPEWEFVNTP 420
                : : * :*.:..:*:..:  : *    ::   :    : : :*:   .: :* :  :::

HDP             QAFTFSPTYKAFLC------ 345
HIV_RT          PLVKLWYQLEKEPIVGAETF 440
                  ..:    :
```

Fig. 2. Sequence alignment between HDP and HIV RT (1RTD).

of 2 fs. The non-bonded list was generated using an atom-based cut-off of 8 Å. The long-range electrostatic interactions were handled by the particle-mesh Ewald algorithm (Darden et al. 1999). The cut-off employed to the Lennard-Jones interaction was 1.4 nm. During the simulations, all bond lengths containing hydrogen atoms were constrained utilizing the Lincs algorithm (Lindahl et al. 2001). The trajectory snapshots were stored for structural analysis at every ps. Root mean square deviation (RMSD), root mean square fluctuation (RMSF) and H-bonds formed between protein and drug was analyzed through GROMACS utilities g_rms, g_rmsf and g_hbond, respectively.

Results and discussion

Homology modelling and flexibility analysis

The homology model of HDP was constructed using the 1RTD (Huang et al. 1998) as a template. The sequence alignment between HDP and HIV RT (1RTD) is shown in Figure 2. Figure 3 shows the Ramachandran plot for the modelled HDP. This plot denotes the distribution of the φ and ψ dihedral angles for the amino acid residues. A total of 73.7% of the residues was found in the most favoured regions, 21.8% in additionally al-

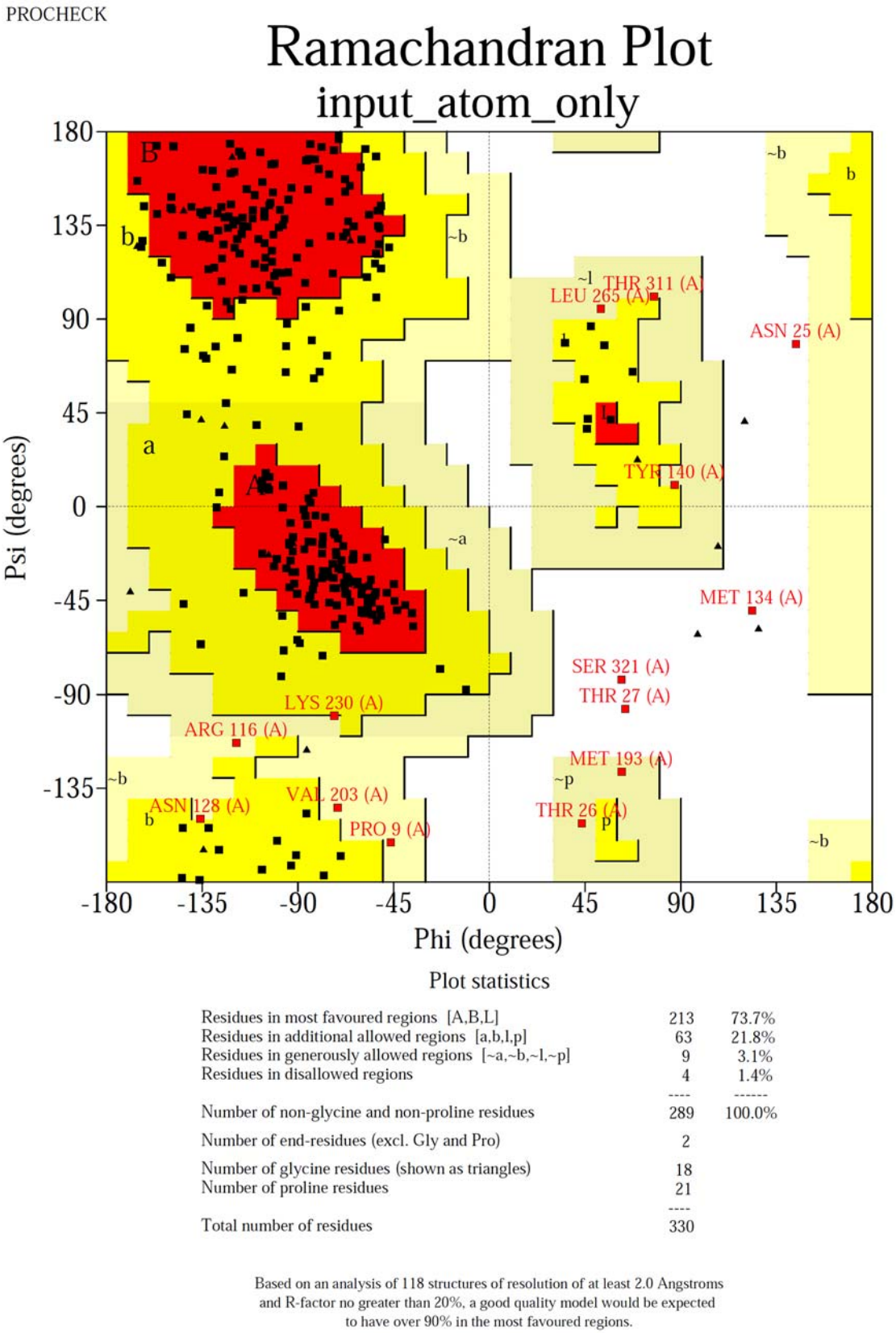

Fig. 3. Ramachandran plot of homology model of HDP.

lowed regions, and 3.1% in the generously allowed regions. Only 1.4% of the residues were found in the disallowed regions. Furthermore, it should be pointed out that none of the binding site residues falls in disallowed regions and residues in disallowed regions are far from the YMDD catalytic motif. This evidently shows that these residues in disallowed regions may not affect the ligand binding to the target.

Table 1. Binding site amino acid residue flexibility by means of normalized mean square displacement.

No.	Binding site residues	<R^2> in native type[a]	<R^2> in mutant type[a,b]
1	Y203	0.0094	0.0086
2	M204/V204	0.0094	0.0084
3	D205	0.0077	0.0070
4	D206	0.0085	0.0078

[a] Normalized mean square displacement.
[b] Amino acids with decreased flexibility of mutant compared to wild structure.

Table 2. Binding free energy analysis of lamivudine with native and mutant type of HBV polymerase.[a]

No.	Complex type	Docking 1	Docking 2	Docking 3	Average
1	Native type HBV polymerase-lamivudine	–8.90	–8.85	–8.70	–8.81
2	Mutant type HBV polymerase-lamivudine	–6.54	–6.86	–6.43	–6.61

[a] All values are in kcal/mol.

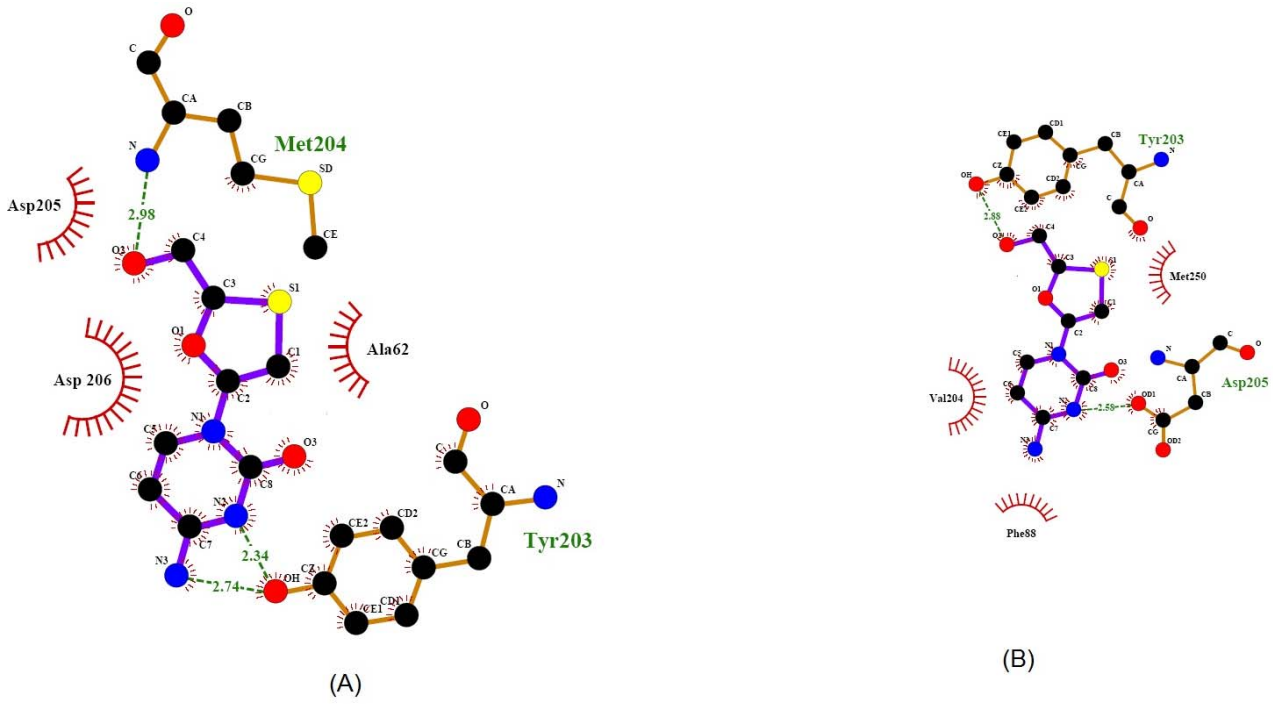

Fig. 4. The interaction of lamivudine with native type HDP (A) and mutant type HDP (B).

Then we computed the <R2> values with the aid of low frequency normal mode of Elnemo program (Suhre & Sanejouand 2004) in order to evaluate the flexibility of the binding residues both in the native and mutant type HDP. The <R2> values of all the amino acid residues present in the binding pocket of HDP are given in Table 1. It is clear that <R2> of amino acids in drug binding pocket of mutant HDP was lower than <R2> of the amino acids in drug binding pocket of native HDP. This certainly indicates that the mutation in the position 204 alters the flexibility of the binding pocket residues. This difference in flexibility affects the binding site conformation of the mutant protein, thereby makes the ligand incapable of binding to the target (Rajasekaran et al. 2010). This results in the decreased binding affinity of lamivudine to mutant-type HDP.

Computation of docking energy

GROMACS package 4.5.3 was used to generate the energy-minimized structure of native and mutant type (Spoel et al. 2005; Hess et al. 2008). One thousand steps of steepest descent energy minimization were carried out for both native and the M204V structures. Subsequently, docking simulation was performed using AutoDock 4.2. In order to evaluate the accurate analysis of binding affinity, the recent trend in the field is the introduction of multiple docking analysis. Subsequently, the results from the different docking were combined to balance the errors in the *in silico* prediction. The result is shown in Table 2, which clearly indicates the binding affinity of lamivudine was higher in the native (–8.81 kcal/mol) compared to the mutant (–6.61 kcal/mol) HDP. This result supports the ineffectual binding of lamivudine with the mutant HDP.

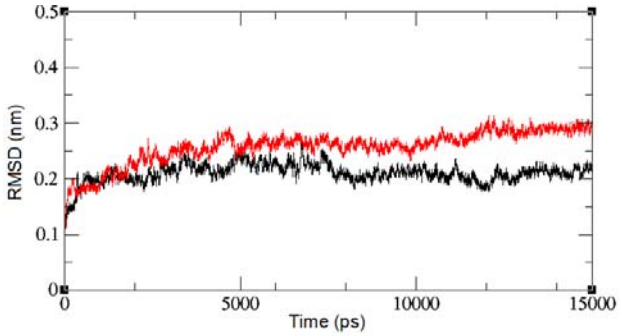

Fig. 5. Root mean square deviations correspond to native (black) and mutant (red) type HDP along the MD simulation at 300 K.

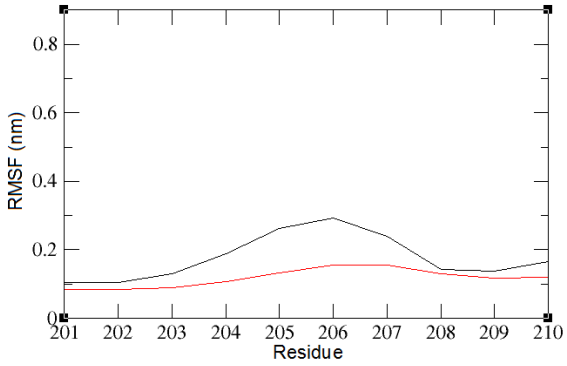

Fig. 6. Root mean square fluctuation of the backbone Cα of mutation nearby residues in the native (black) and mutant (red) type HDP over the entire simulation time.

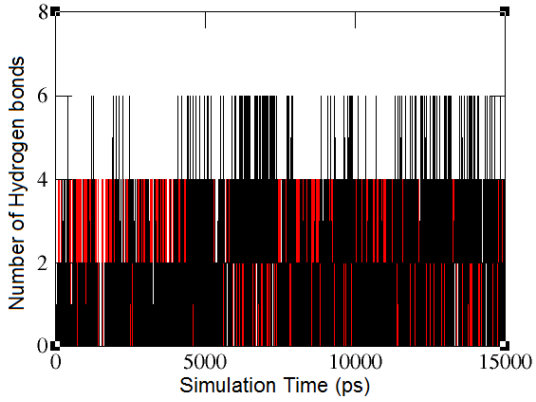

Fig. 7. Hydrogen bonds observed between the protein and ligand along the MD simulation. The symbol coding scheme is as follows: native (black) and mutant (red).

In order to explore the mechanism of resistance, intermolecular interactions in the complex structures were examined with the help of LIGPLOT program. This result is shown in Figure 4. Figure 4A shows that O2 of lamivudine makes hydrogen bond interaction with M204 and N3, N2 of lamivudine make hydrogen bond interaction with Y203 in native HDP-lamivudine complex. Similarly in the mutant (M204) HDP-lamivudine complex, O3 of lamivudine forms hydrogen bond interactions with Y203 and N2 of lamivudine makes a hydrogen bond interaction with D205, but there is no intermolecular hydrogen bond interaction observed in the 204 position. The available evidences suggest that lamivudine binds at a pocket on the surface of the polymerase formed partly by the M204 residue (Fung et al. 2011). Hence, we confirmed that the absence of hydrogen bond interaction at this residue may alter the drug binding mechanism. This revealed that lamivudine was found to adopt the most promising conformation to the native type by identifying M204 as a prospective partner for making hydrogen bond as compared to the mutant type. Furthermore, MD study was carried out to gain insight into the lamivudine binding mechanism.

Molecular dynamics simulation

The MD simulations were performed with the native and mutant HDP-lamivudine complexes. Analysis of Figure 5 shows that the structure reached the equilibrium and does not exhibit any asymptotic behaviour that could indicate unfolding of the structure. The structure is stable during simulation of 15 ns dynamics simulation. RMSD analysis can give an idea of how much the three-dimensional structure has fluctuated over time as well as allowed monitoring local fluctuations. Figure 5 shows the RMSD values of native and mutant HDP-lamivudine complex structures over the simulation time. It is clear that both the native and mutant HDP-lamivudine complex structures exhibited significant deviations from their starting structure. After 13 ns simulation the native complex attained ∼0.2 nm and mutant complex attained ∼0.25 nm. This brings to the conclusion that during the simulation, native HDP-lamivudine complex deviated less compared to the mutant HDP-lamivudine complex. In addition, we have also analyzed the fluctuation behaviour of mutation nearby residues (i.e. from position 201 to 210) using the RMSF analysis in the native and mutant type of HDP (Fig. 6). Analysis of the fluctuations revealed that the mutant showed a less degree of fluctuations when compared to the native type. This difference in fluctuation was further validated by the number of hydrogen bonds analysis during MD simulation (Fig. 7). The number of hydrogen bonds analysis suggests that native HDP-lamivudine complex shows 6 intermolecular hydrogen bonds, whereas in the mutant HDP-lamivudine complex, only 4 intermolecular hydrogen bonds were observed throughout the simulation time. These results suggest that the mutant HDP-lamivudine has decreased binding affinity and loss of crucial hydrogen bond interaction, thus conferring resistance to lamivudine.

Conclusions

In conclusion, the study was done in an effort to understand the drug resistance mechanism of HBV with the aid of molecular docking and MD approaches. Docking study and intermolecular interactions analysis suggested that M204 is the key residue for stable binding of lamivudine to HDP. Furthermore, the fluctuation analysis using $<R^2>$, RMSD and RMSF revealed that mu-

tant exhibits a higher degree of fluctuations compared to native-type HDP. In addition, the hydrogen bond formed between HDP and lamivudine was significantly decreased in mutant HDP-lamivudine complex. This study will help to understand the drug resistant mechanisms and may aid in developing new potent drugs.

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