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Virological response and antiviral resistance mutations in chronic hepatitis B subjects experiencing entecavir therapy: An Indian subcontinent perspective

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ABSTRACT

Entecavir is one of the therapeutic options currently available for the management of chronic hepatitis B. In this study, we aimed to analyse the virological response and antiviral resistance mutations in chronic hepatitis B subjects experiencing entecavir therapy from the Indian subcontinent. A total of 45 chronic hepatitis B subjects were studied at baseline and were followed up on entecavir treatment. Among these subjects, 25 (56%) were HBeAg-positive at baseline. Virological response was measured by hepatitis B virus (HBV) DNA levels. HBV reverse transcriptase (rt) domains were sequenced for the identification of resistance mutations. Three-Dimensional (3D) model of HBV polymerase/rt protein, docking and molecular dynamics simulation (MDS) studies were performed for characterization of antiviral resistance mutations. At the median treatment duration of 6 (IQR 6–11) months, 38 (84%) showed virological response. Subjects who showed anti-HBe response demonstrated significant association with virological response ($p = 0.034$). On sequence analysis, none of the subjects were identified with signature entecavir resistance mutations. However, one subject was exclusively detected with rtV173L mutation. Molecular modeling, docking and MDS studies revealed that the rtV173L mutation cannot confer resistance to entecavir independently. Our findings also showed that the prevailing HBV genotypes, subgenotypes and HBsAg subtypes in this population does not influence treatment outcome to entecavir therapy. In conclusion, entecavir is a potent drug in terms of viral DNA suppression. In addition, none of the subjects developed antiviral resistance mutations to entecavir. Therefore entecavir is a suitable drug of choice in the management of chronic HBV.

1. Introduction

Globally, an estimated two billion people are infected with hepatitis B virus (HBV) and around 350 million live with chronic infection (World-Health-Organisation). Cirrhosis and hepatocellular carcinoma (HCC) are two major long-term sequelae of chronic hepatitis B. The cumulative probability of subjects with chronic hepatitis B developing cirrhosis is 15–20% at the end of 5 years (Fattovich et al., 1991; Liaw et al., 1988). The cumulative incidence of HCC in subjects with chronic hepatitis B without cirrhosis is 1–3% and in subjects with compensated cirrhosis is 10–17% at the end of 5 years (Fattovich et al., 2008). This illustrates the importance of antiviral therapy in controlling disease progression in subjects diagnosed with chronic hepatitis B.

There are several options for the treatment of chronic hepatitis B infection including two formulations of interferon, i.e., standard interferon alfa-2b (IFN-α-2b) and pegylated interferon alfa-2a (peg-IFN-α-2a) and 5 nucleos(t)ide analogues, i.e., lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir. Unlike IFN, nucleos(t)ide analogues require long term and continuous treatment. Over time, the virus evolves strategies to counteract the drug related selection pressure and thereby escapes the antiviral action. Therefore, antiviral resistance is a clinically relevant issue in the therapeutic monitoring of patients with chronic hepatitis B (Hoofnagle et al., 2007; Lok et al., 2007; Lok and McMahon, 2009; EASL, 2009).

In March 2005, entecavir was approved by the Food and Drug Administration (FDA) for the treatment of chronic HBV. It is a guanosine analogue that competes with the natural substrate deoxyguanosine triphosphate and inhibits the reverse transcriptase (rt) activity (Langley et al., 2007). Entecavir is structurally distinct from the other oral drugs and allows incorporation of additional

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nucleotides before chain termination. Entecavir displays activity against the priming function and has been shown to affect both the positive and negative strand DNA synthesis (Seifer et al., 1998).

Entecavir resistance mutations require combinations of substitutions at positions rtI169T, rtV173L, rtL180 M, rtT184G, rtS202I, rtM204 V/I or rtM250 V (Colonno et al., 2006; Tenney et al., 2004). The cumulative incidence of entecavir resistance at 1, 2, 3, 4, 5 and 6 years was found to be 0.2%, 0.5%, 1.2%, 1.2%, 1.2% and 1.2% respectively (Tenney et al., 2009a,b; Yuen et al., 2011). The adverse effects in patients experiencing entecavir over median treatment duration of 184 weeks (3.54 years) were $\leqslant$10% (Manns et al., 2012). Together, with the low adverse effects and high barrier to resistance and long term safety, entecavir was shown to be the suitable drug of choice in management of chronic hepatitis B.

There is paucity of data on the antiviral efficacy of entecavir therapy and the resistance mutations associated with treatment failure in the Indian subcontinent subjects. Therefore, we aimed to analyse the role of entecavir in therapeutic management of chronic hepatitis B in this population. The role of HBV genotypes, subgenotypes, HBsAg subtypes prevailing in this population and its influence on therapeutic outcome was also studied.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Institutional Review Board of Christian Medical College, Vellore (EC Min. No. IRB-10-16-01-2008) and informed written consent was obtained from all the subjects.

2.2. Study subjects

A total of 45 entecavir-experienced subjects were studied. All subjects received entecavir at a standard dosage of 0.5 mg/day. The study subjects comprised of individuals attending the liver clinic of Christian Medical College, a tertiary care teaching hospital in Vellore, South India. These subjects were referred to the department of Clinical Virology for HBV DNA testing and were recruited between January 2007 and November 2011.

The inclusion criteria were treatment-naïve chronic HBV subjects on uninterrupted entecavir treatment. Treatment compliance was checked by verbal questioning and by reviewing the clinical records. The exclusion criteria were history of previous treatment with other HBV antivirals and immunomodulators; add-on or combination therapy; infection with hepatitis C virus (HCV), hepatitis D virus (HDV) or human immunodeficiency virus (HIV); use of immunosuppressive drugs and chemotherapy.

Blood samples were collected in subjects meeting these criteria; plasma was separated and was stored in −60 °C until further analysis.

2.3. Biochemical tests

Serum alanine transaminase (ALT) and serum aspartate transaminase (AST) levels were obtained from the subject's hospital records.

2.4. Serology markers

Hepatitis B surface antigen (HBsAg) was tested in ARCHITECT (Abbott, Weisbaden, Germany) or Monolisa HBsAg ULTRA (Bio-Rad, Marnes-la-coquette, France). HBeAg and anti-HBe testing was performed in an enzyme immunoassay (EIA) (Diasorin S.P.A., Saluggia, Italy). HCV antibody (Ab), HDV Ab and HIV were screened in Ortho HCV 3.0 (Ortho Clinical Diagnostics, Raritan, N.J., USA), IgM anti-HDV EIA (Diasorin S.P.A., Saluggia, Italy) and ARCHITECT HIV Ag/Ab combo (Abbott, Weisbaden, Germany) respectively. The manufacturer's instruction was strictly followed for all the procedures.

2.5. DNA isolation

DNA was extracted from 200 μl of blood plasma using the QIAamp DNA blood MiniKit (Qiagen GmbH, Hilden, Germany). All steps were performed according to the manufacturer's instruction except with the modification in final elution of 50 μl.

2.5.1. HBV DNA quantification

HBV DNA was quantified using artus® HBV RG PCR (Qiagen GmbH, Hilden, Germany) in the Rotor-Gene 3000 or 6000 platform (Corbett Research, Mortlake, Australia). The manufacturer's claimed lower limit of detection (LLD) is 20 IU/mL. The LLD in consideration of DNA purification with Qiamp DNA Blood Mini kit according to our determination was 82 IU/mL (95% detection limit) (Ismail et al., 2011).

2.5.2. HBV polymerase/rt gene amplification and sequencing

HBV polymerase gene covering the entire rt region was amplified (1323 bp) using Platinum® *Taq* DNA polymerase high fidelity (Invitrogen, Carlsbad, Calif., USA). The amplified PCR products were purified and sequenced as described previously (Ismail et al., 2012). Obtained bidirectional sequences were analysed using BioEdit v7.0.9 and the consensus sequence was generated.

2.5.3. HBVrt sequence database

The generated sequences were submitted to the HBVSeq program for HBV drug resistance in Stanford database (<http://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html>) (Rhee et al., 2010). The database assigns a genotype to each sequence and compares the amino acid sequence to the corresponding consensus reference genotype amino acid sequence. The results are then displayed showing the difference between the submitted sequence and the database consensus sequences. HBV amino acid substitutions rtI169T, rtV173L, rtL180M, rtT184G, rtS202I, rtM204V/I or rtM250V were interpreted as entecavir resistance mutations (Colonno et al., 2006; Tenney et al., 2004).

Nucleotide sequences generated from this analysis have been deposited in GenBank database (accession numbers showed in Supplementary material).

2.5.4. Determination of HBV genotypes, subgenotypes and HBsAg subtypes

HBV genotypes were determined by HBVrt sequence analysis in the Standford database.

The study sequences were aligned with published sequences representing all known HBV subgenotypes (Ghosh et al., 2010; Lazarevic et al., 2007; Lusida et al., 2008; Norder et al., 2004; Schaefer et al., 2009). Multiple sequence alignment was performed using the built-in CLUSTALW integrated in MEGA4 (Tamura et al., 2007). HBV subgenotypes were determined by phylogenetic analysis in MEGA4 using the neighbour joining method with a bootstrap test of 1,000 replicates and maximum composite likelihood algorithm.

A new programme for HBsAg subtype determination was developed in Microsoft Visual Basic (VB6). The subtypes were predicted by the subtype programme that examines every combination of surface gene codons at position 122,160,127,159 and 140 (in this order) as deduced by Purdy et al. (2007). The HBsAg subtyping

programme was validated by comparing the results generated by this tool with our earlier published HBsAg subtypes (Ismail et al., 2012). The predicted subtypes are henceforth referred as 'cognate' HBsAg subtypes.

2.6. Homology model of hepatitis B virus polymerase/rt

In the absence of crystallographic structure of desired protein, homology modeling is the potential method to build its three dimensional structure based on suitable protein template (Hillisch et al., 2004). A homology model of HBVrt was built in MODELLER 9v8 using the crystal structure of HIV-1rt template (Protein Data Bank, PDB code: 1RTD A) (Das et al., 2001). Treatment-naive (baseline) and treatment-experienced HBVrt sequences (GenBank accession: JQ514313 and JQ514535) from the study subject was used for the construction of wild type and mutant models respectively. The HBVrt nucleotide sequences were translated into the amino acid sequences using BioEdit v7.0.9. The translated target sequence was aligned with the HIV-1rt template using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The target-template alignment was used to build the three-dimensional model of target protein. Five models were generated and the model with lowest Discrete Optimization Protein Energy (DOPE) was selected for further analysis. The structure validation was performed in PROCHECK using the Structure Analysis and Verification Server (<http://nihserver.mbi.ucla.edu/SAVES/>) for PROCHECK (Laskowski et al., 1996) and VERIFY3D (Eisenberg et al., 1997).

2.6.1. Molecular docking studies

To the modelled protein, the two magnesium (Mg^{2+}) ions and the template primer DNA duplex [d(GCXCCGGCGCTC)-d(GAGCGCCGG)] were located based on the co-ordinates of PDB: 1RTD chain A of HIV-1rt. The 'X' in the DNA duplex was substituted to the complementary base of the rt inhibitor entecavir (guanosine analogue, X = C). Autodock (v1.5.2) was used to dock the HBV polymerase/rt with entecavir. All the possible torsion angles in the ligand molecules were set to rotate freely and polar hydrogen molecules were added. Kollman united atom partial charges were assigned for the receptor. Grid box was generated at the centre of the protein with the grid box size of 40 Å each for *x*, y and *z* respectively. Default Lamarckian genetic algorithm was used for docking analysis and the best docking complex was identified using root mean square deviation (RMSD) cluster analysis. Best binding pose was identified by binding free energy. PyMOL molecular visualisation tool was used to analyse the interactions between entecavir and the target protein (Seeliger and de Groot, 2010).

2.6.2. Molecular dynamics simulation

Molecular dynamics simulation (MDS) was performed for the wild type and mutant HBV polymerase/rt and entecavir complex using GROMACS 4.5.3 (Hess, 2009; Van der Spoel et al., 2005). Gromacs96 force field was applied to the system. Topology files and forcefield parameter for ligands was generated using PRODRG server (Schuttelkopf and van Aalten, 2004). The protein–ligand complex was placed at the centre of the cubic box and solvated by water molecules. The system had an initial net charge of 2.200000e + 01 kcal/mol. The whole system was brought to neutral by adding 22 Cl^- ions. The Berendsen temperature and pressure were used to maintain constant temperature (300 K) and pressure. The Particle Mesh Ewald (PME) was used for long range electrostatistics and LINCS algorithm were used for the bond constraint (Abraham and Gready, 2011; Hess, 2008). Finally 5000 ps MDS were performed for both wild and mutant complex. The trajectory was visually inspected using PyMol and 2D graphs were generated using XMGRACE for protein backbone RMSD, Ligand positional RMSD and Root Mean Square Fluctuation (RMSF).

2.7. Statistical analysis

Medians with interquartile range (IQR) were used to describe variables with skewed distribution. Comparison of study variables was done using non-parametric tests; Mann-Whitney U test, Kruskal–Wallis test, Chi-square test or Wilcoxon signed-rank test as appropriate. A *p*-value of <0.05 was considered statistically significant. All analysis was done using STATA 11 (StataCorp, College Station, Tex., USA). Graphs were generated using Graphpad Prism v5.0 (GraphPad Software, San Diego, California, USA).

3. Results

A total of 45 entecavir-experienced subjects were studied at baseline and followed-up with entecavir treatment. Among these subjects, 42 (93.3%) were male and 3 (6.7%) were female; their median age was 34 (IQR 25–50) years.

3.1. Baseline characteristics

Among the 45 subjects at baseline, 25 (56%) were HBeAg-positive. One (4%) HBeAg-positive subject in the anti-HBe seroconversion phase and 19 (95%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA levels in HBeAg-positive and negative subjects were 7.3 (IQR 6.48–7.48) and 4.5 (IQR 3.74–5.74) $\log_{10}$ IU/mL respectively ($p = 0.0001$). The median ALT and AST levels were 47 (IQR 30–81) U/L and 37 (IQR 29–64) U/L respectively. None of the subjects were detected with established antiviral resistance mutations at baseline.

3.2. Virological response and antiviral resistance mutations

The median entecavir treatment duration in the subjects was 6 (IQR 6–11) months. All subjects continued to be positive for HBsAg. There existed a significant difference between baseline and on-treatment measurements for HBV DNA, ALT and AST levels respectively (Fig. 1).

Among the 45 subjects, 38 (84%) were classified as responders who showed $\geqslant 1$ $\log_{10}$ IU/ml of HBV DNA reduction with median treatment duration of 6 months ($n = 27$) or undetectable HBV DNA (<82 IU/mL) at the median treatment duration of 12 months ($n = 11$). Seven (16%) were non responders showing <1 $\log_{10}$ IU/

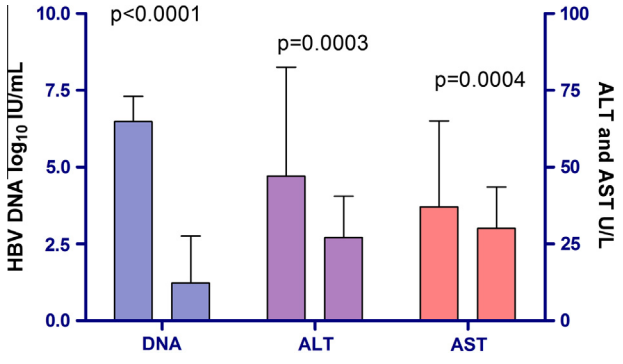

Fig. 1. Differences between pre-treatment (baseline) and on-treatment measurement of study variables. On-treatment measurement is measured at the median entecavir treatment duration of 6 (IQR 6–11) months. Wilcoxon signed-rank was performed and the significance level is shown for each variable respectively. The values are showed in median levels and the error bars indicate interquartile range (IQR).

mL reduction of HBV DNA in median treatment duration of 6 months ($n = 1$) or continued to be positive for HBV DNA after median treatment duration of 12 months ($n = 6$). On analysis of factors associated with virological response, subjects who showed anti-HBe response showed significant association with virological response ($p = 0.034$; Table 1).

In these 45 subjects, 23 were detected positive in real-time PCR with median HBV DNA levels of 2.69 (IQR 2.03–3.53) $\log_{10}$ IU/mL. Among these samples, 13 amplified in HBVrt PCR. The median treatment duration was 6 (IQR 6–7) months and none of the subjects were identified with signature entecavir resistance mutations in combination. However, one subject was exclusively identified with a rtV173L mutation. This subject showed virological response with reduction in HBV DNA level of 1.8 $\log_{10}$ IU/mL at 7 months therapy.

3.3. Baseline factors for prediction of virological response

On analysis of baseline factors, HBeAg and anti-HBe antibody showed significant association for the prediction of virological response ($p = 0.01$ in both; Table 2).

3.4. HBV genotypes, subgenotypes, HBsAg subtypes and entecavir response

HBV genotypes A, C and D were identified in 9 (20%), 9 (20%) and 27 (60%) subjects respectively. On analysis, the response rate to genotype A, C and D were 78%, 78% and 89% respectively. There was no significant difference in proportion of subjects who showed entecavir response and non-response (Table 3a).

All samples identified as genotypes A and C were determined to be subgenotypes A1 and C1 respectively. Among genotype D infected subjects, subgenotypes D1, D2, D3 and D5 were identified in 6, 15, 4 and 2 subjects respectively. When HBV subgenotypes of genotype D subjects were separately analysed, no subgenotype-dependent response or non-response to entecavir was observed (Table 3b).

The cognate HBsAg subtypes *adw2*, *adr*, *ayw2* and *ayw3* were identified in 10 (22%), 9 (20%), 9 (20%) and 17 (38%) subjects respectively. The virological response for the predicted subtypes *adw2*, *adr*, *ayw2* and *ayw3* were 80%, 78%, 89% and 88%, respectively. There was no significant difference in proportion of subjects who showed entecavir response and non-response. (Table 3c).

3.5. Homology modeling

To have an insight into the molecular inhibition of HBV polymerase/rt with entecavir a three dimensional structure of HBV polymerase/rt was constructed based on HIV-1rt template.

3.5.1. Structure validation

The Structural Analysis and Verification Server showed the modelled structure of HBVrt with overall satisfactory structural geometry. Less than 1% of the residues were present in the dis-allowed region (Fig. 2). VERIFY3D results showed 73.1% of the residues with an average 3D-1D score >0.2. This data suggests that our modelled structure is suitable for further analysis.

3.5.2. Effect of rtV173L mutation and entecavir action: molecular modeling and docking studies

To understand the effect of rtV173L mutation and entecavir efficacy, homology model of the mutant HBV polymerase/rt sequence from the treatment experienced subject (GenBank accession:

Table 1
Analysis of on-treatment factors associated with entecavir response. Virological response is measured at the median treatment duration of 6 (IQR 6–11) months.

Variables	Responders ($n = 38$)	Non-responders ($n = 7$)	p Value
ALT (U/L)†	26 (22–36)	29 (19–91)	0.406
AST (U/L)†	31 (23–43)	30 (25–54)	0.802
HBeAg Pos*	18 (47)	6 (86)	0.062
HBeAg Neg*	20 (53)	1 (14)	
Anti-HBe Pos*	22 (58)	1 (14)	0.034
Anti-HBe Neg*	16 (42)	6 (86)	

Values are †median (IQR) or *number (%). Statistical analysis was performed by †Mann–Whitney U test or *Chi-square test.

Table 2
Analysis of baseline factors for prediction of entecavir response.

Variables	Responders ($n = 38$)	Non-responders ($n = 7$)	p Value
Age, years†	38 (25–50)	25 (19–41)	0.240
Gender, male*	35 (92)	7 (100)	0.442
ALT (U/L)†	38 (27–85)	54 (47–63)	0.316
AST (U/L)†	38 (33–44)	37 (28–67)	0.661
HBV DNA ($\log_{10}$ IU/mL)†	5.74 (3.95–7.3)	7.3 (3.95–7.3)	0.266
HBeAg Pos*	18 (47)	7 (100)	0.01
HBeAg Neg*	20 (53)	0	
Anti-HBe Pos*	20 (53)	0	0.01
Anti-HBe Neg*	18 (47)	7 (100)	

Values are †median (IQR) or *number (%). Statistical analysis was performed by †Mann–Whitney U test or *Chi-square test.

Table 3a
Association of HBV genotypes and entecavir response.

Virological response	Genotype A ($n = 9$)	Genotype C ($n = 9$)	Genotype D ($n = 27$)	p Value
Response*	7 (78)	7 (78)	24 (89)	0.602
Non-response*	2 (22)	2 (22)	3 (11)	
Treatment duration (months)†	9 (6–12)	7 (6–10)	6 (6–10)	0.522

Data are †median (IQR) or *number (%). Statistical analysis was performed by †Kruskal–Wallis or *Chi-square tests.

Table 3b
Association of HBV subgenotypes of genotype D and entecavir response.

Virological response	D1 ($n = 6$)	D2 ($n = 15$)	D3 ($n = 4$)	D5 ($n = 2$)	p Value
Response*	6 (100)	13 (87)	3 (75)	2 (100)	0.603
Non-response*	0	2 (13)	1 (25)	0	
Treatment duration (months)†	6 (6–9)	6 (6–12)	8 (6–11)	6 (3–6)	0.533

Data are †median (IQR) or *number (%). Statistical analysis was performed by †Kruskal–Wallis or *Chi-square tests.

Table 3c
Association of predicted HBsAg subtypes and entecavir response.

Virological response	*adw2* ($n = 10$)	*adr* ($n = 9$)	*ayw2* ($n = 9$)	*ayw3* ($n = 17$)	p Value
Response*	8 (80)	7 (78)	8 (89)	15 (88)	0.855
Non-response*	2 (20)	2 (22)	1 (11)	2 (12)	
Treatment duration (months)†	8 (6–12)	7 (6–10)	6 (6–10)	6 (6–12)	0.844

Data are †median (IQR) or *number (%). Statistical analysis was performed by †Kruskal–Wallis or *Chi-square tests.

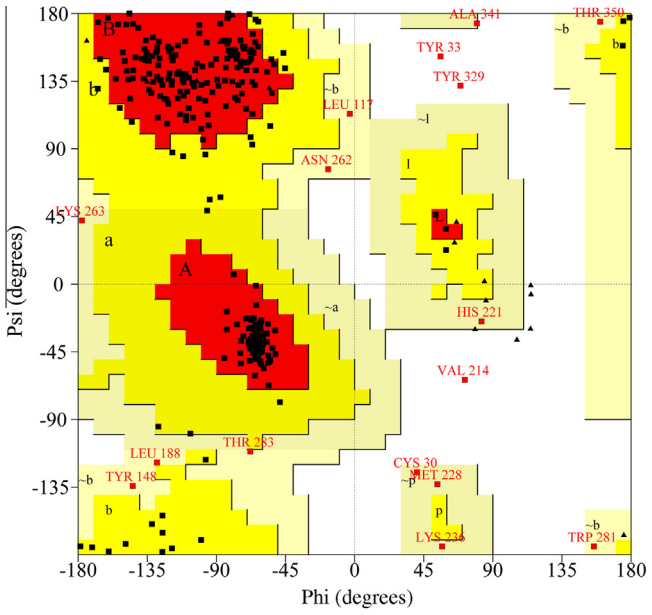

Fig. 2. Ramachandran plot of HBV polymerse/rt model. Ramachandran plot showing the phi (φ)-psi (ψ) torsion angles for all the HBVrt aminoacid residues in the structure. Glycine and proline residues are shown as triangles (▲) and are not restricted to the regions of plots. The distribution of φ, ψ angles showed 83.8% residues in the most favourable core region (shown in red), 11.2% of residues in allowed region (yellow), 4.1% residues in the generous region (third level) and 0.9% residues in the disallowed region. The plot was generated in PROCHECK using the Structure Analysis and Verification Server (<http://nihserver.mbi.ucla.edu/SAVES/>).

JQ514535) was constructed. The model showed that the residue rtV173 was located below the DNA template binding region. The rtV173L mutation caused minor conformational change at the mutation site but did not alter the entecavir binding site or the relative position of rtF88 that interacts with dNTP substrate (Fig. 3). Our docking analysis revealed that the rtV173L mutation decreased the docking score from −6.67 to −5.73 kcal/mol (<1 kcal/mol) and therefore does not show any significant changes in the binding efficacy of entecavir (Table 4).

Table 4
Molecular docking results of wild type and mutant HBV polymerase/rt with entecavir.

Type	Hydrogen bond interactions	H-donor::H-acceptor	Bond length	Docking score (kcal/mol)	No. of H-bonds
HBV rtV173 (wild type)	Asp205	UNK:N1::Asp:205:OD2	3.16 Å	−6.67	5
	Asp206	UNK:N1:: Asp206:OD1	3.30 Å		
	Asp83	UNK:N1:: Asp83: OD2	2.76 Å		
	Asn33	Asn33: ND2:: UNK:O17	3.17 Å		
	Arg41	Arg41:NH2::UNK:O21	3.04 Å		
HBV rtV173L (mutant)	Asp205	UNK:N1::Asp205:OD2	2.77 Å	−5.73	3
	Asn33	UNK:H3O::Asn33:OD1	2.78 Å		
	Asp83	UNK:N3::Asp83:OD2	3.13 Å		

3.5.3. Molecular dynamics simulation

To investigate the dynamic motion of HBV polymerase/rt (wild and mutant), MDS were performed. Root mean square deviation of wild and mutant protein backbones indicate structural fluctuations in the backbone of proteins during the 5 ns trajectory period (Fig. 4a). The backbone of wild and mutant HBV polymerase/rt showed a standard deviation of less than 1 Å and indicates a better quality of simulation. The RMSD graph showed that rtV173L mutation does not affect the conformation and their stability during the simulation period. The ligand positional RMSD analysis throughout the simulation period also showed no significant changes during the trajectory period for both wild and mutant HBV polymerase/rt (Fig 4b). The RMS fluctuation for HBV rtV173 (wild type) and rtV173L (mutant) was 0.2145 nm and 0.2118 nm respectively (Fig. 4c).

4. Discussion

Virological response and antiviral resistance development were the two major outcomes determined in this study. In our analysis of baseline factors that would predict the virological response to entecavir, HBeAg and anti-HBe antibody showed significant association for virological response ($p = 0.01$ in both). All 7 (16%) non-responders were HBeAg-positive and anti-HBe negative. This finding shows that HBeAg-positive subjects have low response rate when compared to HBeAg-negative subjects. This finding is in

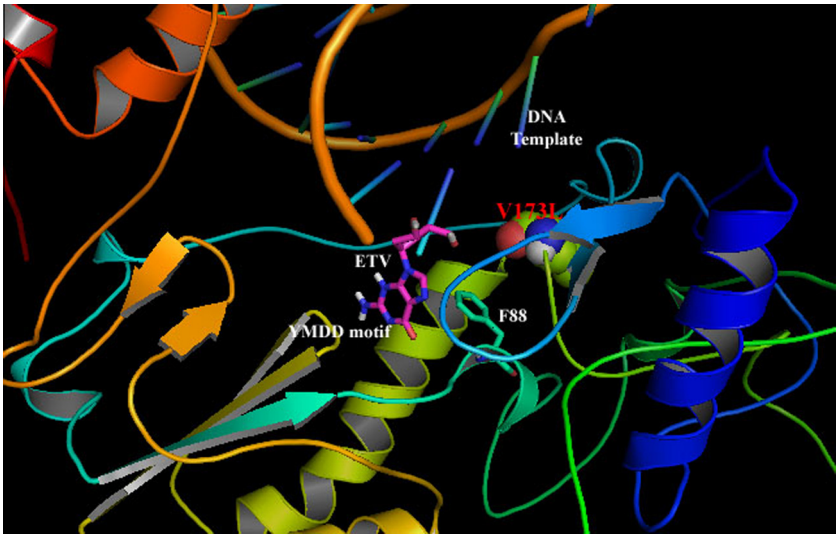

Fig. 3. Effect of rtV173L mutation and entecavir binding. Homology model of HBV polymerase/rt of a study sequence identified with rtV173L mutation was constructed (GenBank accession: JQ514535). The location of rtV173L is shown. Also shown are the YMDD motif and entecavir (ETV) binding sites. The rt amino acid position 173 just lies below the DNA template region and the rtV173L mutation did not appear to alter entecavir binding or rtF88 positioning that interacts with the dNTP substrate.

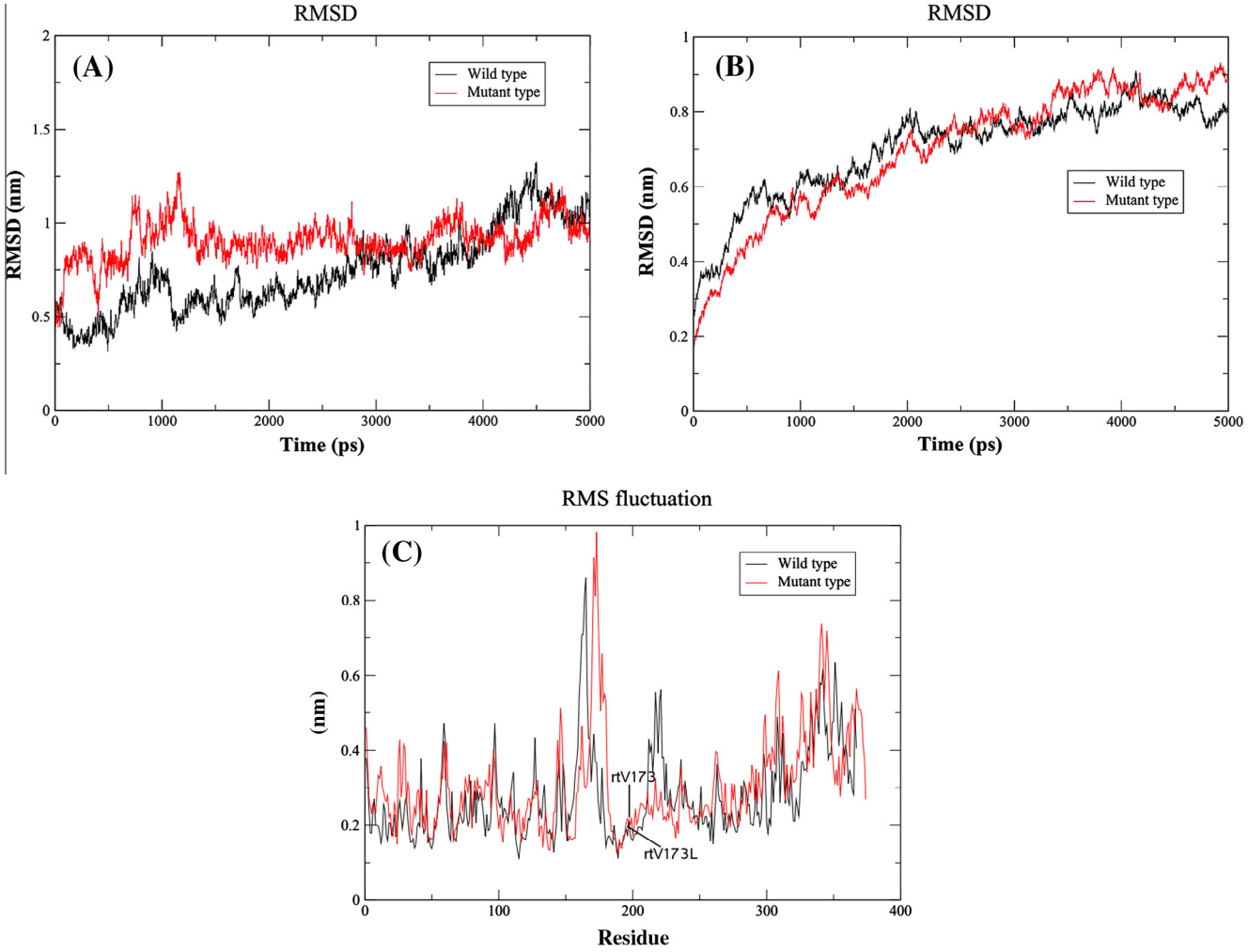

Fig. 4. (A) Protein backbone RMSD graph for wild type and mutant HBV polymerase/rt. In the rtV173L mutant type, RMSD value increased from 0.18 nm to 0.7 nm and fluctuated in the range of 0.1 nm up to 2000 ps. The overall RMSD fluctuation during the trajectory period was negligible between 0.18 nm and 0.90 nm (<1 nm). In wild type, the fluctuation ranged between 0.4 nm and 1.25 nm throughout the MDS period. (B) Positional RMSD graph for wild type and mutant HBV polymerase/rt. Both wild and mutant ligands maintained their RMSD over the time frame. (C) RMS fluctuation of the wild type and mutant HBV polymerase/rt. The wild type HBV polymerase/rt showed sudden increment of RMS fluctuation between rtS135 (0.4059 nm) and rtS143 (0.4308 nm). The maximum fluctuation was observed at rtY141 (0.8609 nm). In mutant HBV polymerase/rt, sudden RMS fluctuation was observed between rtL144 (0.4229 nm) and rtH156 (0.5309 nm). The maximum fluctuation was observed at rtK149 (0.9819 nm).

agreement with the recent study which showed the virological response for HBeAg-negative subjects being comparably higher (98.3%) than HBeAg-positive subjects (82.9%) after 3 years of entecavir treatment (Yuen et al., 2011).

Overall, 84% of subjects responded to entecavir at the median treatment duration of 6 months. The results of our finding broadly agree with a recent study, where 81.1% showed response after 12 months of entecavir treatment (Yuen et al., 2011). On analysis of factors associated with response, subjects with anti-HBe seroconversion showed significant association with entecavir response.

Previously, Lurie et al. (2005) has showed entecavir response to be consistent across HBV genotypes A, B, C and D respectively. In our analysis also there was no significant difference in proportion of subjects who showed response and non-response. This observation was also noticed when subgenotypes of genotype D were separately analysed. This shows that HBV genotypes have no role in predicting entecavir response. There was also no significant association between the predicted HBsAg subtypes and entecavir response. There was no significant difference in treatment duration between the variables analysed and this excludes the chance of bias in the analysis performed. The newly developed tool for HBsAg subtype determination in our study reduces time involved and error rates, caused by manual procedures. Therefore, this tool will have a wider application potential in clinical setting and epidemiological studies.

Previous reports have shown that entecavir resistance occurs by the combination of three or more amino acid substitutions in the HBVrt region. One of the entecavir-experienced subjects in our study had an exclusive rtV173L mutation after 7 months treatment. However, this subject showed virological response with reduction in HBV DNA levels of 1.8 $\log_{10}$ IU/mL from baseline. Delaney et al. (2003) in their molecular modeling studies, showed that rtV173L together with rtL180M and rtM204V mutations alter the rtF88 residue that is crucial for DNA polymerization. We constructed a HBVrt model of the sequence with rtV173L mutation and docked it with entecavir to assess whether this mutation independently impacted on entecavir binding. In our analysis the rtV173L mutation neither altered the entecavir binding nor the relative position of rtF88 that interacts with dNTP. Our molecular docking and molecular dynamics simulation results show that rtV173L mutation alone cannot confer resistance to entecavir or alter the relative amino acid residues exclusively. This collective

information reiterates that solitary rtV173L mutation does not impact the antiviral action of entecavir which is corroborated by the virological response in the study subject. Further follow up samples from this subject were not available to assess the long term outcome.

This study holds some limitations. First, the number of study subjects is limited since entecavir is still sparingly used in this country for the treatment of chronic HBV infection. Secondly, the detection of antiviral resistance mutations increases with the increasing treatment duration. The short duration of study follow-up could have limited the detection of antiviral resistance mutations associated with entecavir therapy. Therefore, a larger sample size with longitudinal analysis is of utmost importance. Third, the LLD of the HBV DNA quantification assay used in our study is 82 IU/mL. This does not meet the currently recommended LLD of 10 IU/mL for therapeutic monitoring (Pawlotsky et al., 2008). However, our estimated response rates concur with those of previous findings (Lai et al., 2006; Pol and Lampertico, 2012; Yuen et al., 2011).

5. Conclusions

In this study, subjects who had spontaneous anti-HBe seroconversion showed better response to entecavir. Overall 84% of entecavir-experienced subjects responded to entecavir with the median treatment duration of 6 months and none developed entecavir resistance mutations. It is very evident from our finding that HBV genotypes (A, C and D), subgenotypes (of genotype D) and HBsAg subtypes do not influence treatment outcome to entecavir therapy. Our molecular modeling, docking and dynamics simulation results reiterated that the rtV173L mutation cannot independently confer resistance to entecavir or alter the relative amino acid residues. Therefore, extending the molecular modeling approach will aid in the identification of the true impact that mutations have on antiviral action. This study would thus expand the choice of antivirals for the treatment of chronic HBV subjects in this population.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.02.012.

References

Abraham, M.J., Gready, J.E., 2011. Optimization of parameters for molecular dynamics simulation using smooth particle-mesh Ewald in GROMACS 4.5. J. Comput. Chem. 32, 2031–2040.

Colonno, R.J., Rose, R., Baldick, C.J., Levine, S., Pokornowski, K., Yu, C.F., Walsh, A., Fang, J., Hsu, M., Mazzucco, C., Eggers, B., Zhang, S., Plym, M., Klesczewski, K., Tenney, D.J., 2006. Entecavir resistance is rare in nucleoside naive patients with hepatitis B. Hepatology 44, 1656–1665.

Das, K., Xiong, X., Yang, H., Westland, C.E., Gibbs, C.S., Sarafianos, S.G., Arnold, E., 2001. Molecular modeling and biochemical characterization reveal the mechanism of hepatitis B virus polymerase resistance to lamivudine (3TC) and emtricitabine (FTC). J. Virol. 75, 4771–4779.

Delaney, W.E.t., Yang, H., Westland, C.E., Das, K., Arnold, E., Gibbs, C.S., Miller, M.D., Xiong, S., 2003. The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. J. Virol. 77, 11833–11841.

EASL Clinical Practice Guidelines: management of chronic hepatitis B, 2009. J. Hepatol. 50, 227–242.

Eisenberg, D., Luthy, R., Bowie, J.U., 1997. VERIFY3D: assessment of protein models with three-dimensional profiles. Methods Enzymol. 277, 396–404.

Fattovich, G., Brollo, L., Giustina, G., Noventa, F., Pontisso, P., Alberti, A., Realdi, G., Ruol, A., 1991. Natural history and prognostic factors for chronic hepatitis type B. Gut 32, 294–298.

Fattovich, G., Bortolotti, F., Donato, F., 2008. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J. Hepatol. 48, 335–352.

Ghosh, S., Banerjee, P., RoyChoudhury, A., Sarkar, S., Ghosh, A., Santra, A., Banerjee, S., Das, K., Dwibedi, B., Kar, S.K., Rao, V.G., Bhat, J.T., Singh, N., Chowdhury, A., Datta, S., 2010. Unique hepatitis B virus subgenotype in a primitive tribal community in eastern India. J. Clin. Microbiol. 48, 4063–4071.

Hess, B., 2008. P-LINCS: a parallel linear constraint solver for molecular simulation. J. Chem. Theory Comput. 4, 116–122.

Hess, B., 2009. GROMACS 4: algorithms for highly efficient, load-balanced, and scalable molecular simulation. Abstracts of Papers of the American Chemical Society 237.

Hillisch, A., Pineda, L.F., Hilgenfeld, R., 2004. Utility of homology models in the drug discovery process. Drug Discov. Today 9, 659–669.

Hoofnagle, J.H., Doo, E., Liang, T.J., Fleischer, R., Lok, A.S., 2007. Management of hepatitis B: summary of a clinical research workshop. Hepatology 45, 1056–1075.

Ismail, A.M., Sivakumar, J., Anantharam, R., Dayalan, S., Samuel, P., Fletcher, G.J., Gnanamony, M., Abraham, P., 2011. Performance characteristics and comparison of Abbott and artus real-time systems for hepatitis B virus DNA quantification. J. Clin. Microbiol. 49, 3215–3221.

Ismail, A.M., Samuel, P., Eapen, C.E., Kannangai, R., Abraham, P., 2012. Antiviral resistance mutations and genotype-associated amino acid substitutions in treatment-naive hepatitis B virus-infected individuals from the Indian subcontinent. Intervirology 55, 36–44.

Lai, C.L., Shouval, D., Lok, A.S., Chang, T.T., Cheinquer, H., Goodman, Z., DeHertogh, D., Wilber, R., Zink, R.C., Cross, A., Colonno, R., Fernandes, L., 2006. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. N. Engl. J. Med. 354, 1011–1020.

Langley, D.R., Walsh, A.W., Baldick, C.J., Eggers, B.J., Rose, R.E., Levine, S.M., Kapur, A.J., Colonno, R.J., Tenney, D.J., 2007. Inhibition of hepatitis B virus polymerase by entecavir. J. Virol. 81, 3992–4001.

Laskowski, R.A., Rullmannn, J.A., MacArthur, M.W., Kaptein, R., Thornton, J.M., 1996. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J. Biomol. NMR 8, 477–486.

Lazarevic, I., Cupic, M., Delic, D., Svirtlih, N.S., Simonovic, J., Jovanovic, T., 2007. Distribution of HBV genotypes, subgenotypes and HBsAg subtypes among chronically infected patients in Serbia. Arch. Virol. 152, 2017–2025.

Liaw, Y.F., Tai, D.I., Chu, C.M., Chen, T.J., 1988. The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. Hepatology 8, 493–496.

Lok, A.S.F., McMahon, B.J., AASLD Practice Guidelines. Chronic Hepatitis B: Update 2009. <http://www.aasld.org/practiceguidelines/Documents/Bookmarked%20Practice%20Guidelines/Chronic_Hep_B_Update_2009%208_24_2009.pdf> (accessed 10.03.12).

Lok, A.S., Zoulim, F., Locarnini, S., Bartholomeusz, A., Ghany, M.G., Pawlotsky, J.M., Liaw, Y.F., Mizokami, M., Kuiken, C., 2007. Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. Hepatology 46, 254–265.

Lurie, Y., Manns, M.P., Gish, R.G., Chang, T.T., et al., 2005. The efficacy of entecavir is similar regardless of disease-related baseline subgroups in treatment of nucleoside-naive, HBeAG(+) and HBeAg(−) patients with chronic hepatitis B. J. Hepatol. 42, S184.

Lusida, M.I., Nugrahaputra, V.E., Handajani Soetjipto, R., Nagano-Fujii, M., Sasayama, M., Utsumi, T., Hotta, H., 2008. Novel subgenotypes of hepatitis B virus genotypes C and D in Papua, Indonesia. J. Clin. Microbiol. 46, 2160–2166.

Manns, M.P., Akarca, U.S., Chang, T.T., Sievert, W., Yoon, S.K., Tsai, N., Min, A., Pangerl, A., Beebe, S., Yu, M., Wongcharatrawee, S., 2012. Long-term safety and tolerability of entecavir in patients with chronic hepatitis B in the rollover study ETV-901. Expert Opin. Drug Saf. 11, 361–368.

Norder, H., Courouce, A.M., Coursaget, P., Echevarria, J.M., Lee, S.D., Mushahwar, I.K., Robertson, B.H., Locarnini, S., Magnius, L.O., 2004. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 47, 289–309.

Pawlotsky, J.M., Dusheiko, G., Hatzakis, A., Lau, D., Lau, G., Liang, T.J., Locarnini, S., Martin, P., Richman, D.D., Zoulim, F., 2008. Virologic monitoring of hepatitis B virus therapy in clinical trials and practice: recommendations for a standardized approach. Gastroenterology 134, 405–415.

Pol, S., Lampertico, P., 2012. First-line treatment of chronic hepatitis B with entecavir or tenofovir in 'real-life' settings: from clinical trials to clinical practice. J. Viral. Hepat. 19, 377–386.

Purdy, M.A., Talekar, G., Swenson, P., Araujo, A., Fields, H., 2007. A new algorithm for deduction of hepatitis B surface antigen subtype determinants from the amino acid sequence. Intervirology 50, 45–51.

Rhee, S.Y., Margeridon-Thermet, S., Nguyen, M.H., Liu, T.F., Kagan, R.M., Beggel, B., Verheyen, J., Kaiser, R., Shafer, R.W., 2010. Hepatitis B virus reverse transcriptase sequence variant database for sequence analysis and mutation discovery. Antiviral. Res. 88, 269–275.

Schaefer, S., Magnius, L., Norder, H., 2009. Under construction: classification of hepatitis B virus genotypes and subgenotypes. Intervirology 52, 323–325.

Schuttelkopf, A.W., van Aalten, D.M.F., 2004. PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. Acta Crystallogr. D-Biol. Crystallogr. 60, 1355–1363.

Seeliger, D., de Groot, B.L., 2010. Ligand docking and binding site analysis with PyMOL and Autodock/Vina. J. Comput. Aided Mol. Des. 24, 417–422.

Seifer, M., Hamatake, R.K., Colonno, R.J., Standring, D.N., 1998. In vitro inhibition of hepadnavirus polymerases by the triphosphates of BMS-200475 and lobucavir. Antimicrob. Agents Chemother. 42, 3200–3208.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Tenney, D.J., Levine, S.M., Rose, R.E., Walsh, A.W., Weinheimer, S.P., Discotto, L., Plym, M., Pokornowski, K., Yu, C.F., Angus, P., Ayres, A., Bartholomeusz, A., Sievert, W., Thompson, G., Warner, N., Locarnini, S., Colonno, R.J., 2004. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. Antimicrob. Agents Chemother. 48, 3498–3507.

Tenney, D.J., Pokornowski, K.A., Rose, R.E., Baldick, C.J., Eggers, B.J., Fang, J., Wichroski, M.J., Diva, U.A., Xu, D., Wilber, R.B., Brett-Smith, H., Iloeje, U.H., 2009a. Entecavir maintains a high genetic barrier to HBV resistance through 6 years in naive patients. J. Hepatol. 50, S10.

Tenney, D.J., Rose, R.E., Baldick, C.J., Pokornowski, K.A., Eggers, B.J., Fang, J., Wichroski, M.J., Xu, D., Yang, J., Wilber, R.B., Colonno, R.J., 2009b. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naive patients is rare through 5 years of therapy. Hepatology 49, 1503–1514.

Van der Spoel, D., Lindahl, E., Hess, B., Groenhof, G., Mark, A.E., Berendsen, H.J.C., 2005. GROMACS: fast, flexible, and free. J. Comput. Chem. 26, 1701–1718.

World-Health-Organization, August 2008 Revision. Hepatitis B. Fact Sheet No. 204. <http://www.who.int/mediacentre/factsheets/fs204/en/> (accessed 01.12.11).

Yuen, M.F., Seto, W.K., Fung, J., Wong, D.K., Yuen, J.C., Lai, C.L., 2011. Three years of continuous entecavir therapy in treatment-naive chronic hepatitis B patients: VIRAL suppression, viral resistance, and clinical safety. Am. J. Gastroenterol. 106, 1264–1271.