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Understanding the Unique Mechanism of L-FMAU (Clevudine) against Hepatitis B Virus: Molecular Dynamics Studies

Youhoon Chong and Chung K. Chu*

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602, USA

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Abstract—The molecular dynamics simulation of HBV-polymerase·DNA·L-FMAU-TP complex demonstrated that L-FMAU-TP may not serve as a substrate for HBV polymerase because the appropriate binding of L-FMAU-TP to the active site of HBV polymerase may not take place without the unfavorable conformational adjustment, which prevents L-FMAU-TP from being incorporated into the growing viral DNA chain.

In the last several years, active pursuit of effective anti-hepatitis B virus (HBV) agents has resulted in the identification of a number of potentially useful nucleoside analogues. Treatment with nucleosides has shown immediate clinical benefits such as reduced viral load, suppression of progression of liver disease, and induction of immunological clearance or seroconversion. L-FMAU (clevudine) was reported by Chu et al.[1] as a potent antiviral agent against HBV (EC_{50} 0.1 μM in HepG2 2.2.15 cells) as well as EBV, which has low cytotoxicities in a variety of cell lines including MT2, CEM, H1 and HepG2 2.2.15 and bone marrow progenitor cells. L-FMAU is metabolized in cells by the cellular thymidine kinase as well as deoxycytidine kinase to its monophosphate, and subsequently to the di- and triphosphate.[2] L-FMAU is currently undergoing phase II clinical trials in patients who are chronically infected with HBV. Hepadnaviruses replicate by a multistep mechanism that begins with reverse transcription of pregenomic RNA. The DNA synthesis is initiated by a primer which directly binds the first nucleotide (dGTP) of the DNA minus strand.[3] The DNA plus strand is then synthesized with the minus strand as a template to yield the mature, partially double-stranded DNA virus. Therefore, understanding how and which of these three distinct phases of hepadnaviral replication (priming, reverse transcription and DNA-dependent DNA synthesis) can be blocked by nucleoside analogues, can provide valuable information for the discovery of more potent and safe anti-HBV drugs. Even though L-FMAU is known to act specifically on viral DNA synthesis, and its triphosphate inhibits the HBV DNA synthesis in a dose-dependent manner[4] without being incorporated into the DNA or chain termination,[5] the precise understanding of the mechanism of action of L-FMAU at the polymerase level has not been realized. It is known that nucleoside inhibitors, in general, interfere with the viral polymerase activities by both competitive inhibition and incorporation to the viral DNA strands.[6] Most of the antiviral nucleoside analogues studied thus far, with the exception of ribavirin,[7] exerted their antiviral action through the inhibition of viral polymerase and their incorporation into the viral DNA. By using replicating cores extracted from congenitally infected ducks, the anti-HBV mechanism of 3TC-TP was determined to be the inhibition of virus replication by acting as a chain terminator of both the RT and DNA polymerase activities of the enzyme.[8] Also, even though entecavir and lobucavir are not obligate chain terminators of DNA synthesis by virtue of the OH group content of their sugar moieties, endogenous sequencing reactions conducted in replicative HBV nucleocapsids suggested that they may act as chain terminators by introducing enough structural distortion to preclude the enzyme from optimal interaction with the 3′ end of the growing DNA chain.[9] It was found that L-FMAU-TP is not a substrate of EBV DNA polymerase, which suggests that the anti-EBV activity of L-FMAU may

*Corresponding author. Tel.: +1-706-542-5379; fax: +1-706-542-5381; e-mail: dchu@mail.rx.uga.edu

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not be due to its incorporation into EBV DNA.[10] Therefore, of considerable mechanistic interest is how L-FMAU-TP can competitively inhibit the HBV polymerase,[4] without being incorporated into the growing HBV-DNA chain. For this purpose, L-FMAU-TP/HBV polymerase complex was constructed and simulated by molecular dynamics to obtain the structural as well as mechanistic information of L-FMAU-TP in a molecular level.

Methods

The three-dimensional structural information of HBV polymerase is still not available. However, the recently published homology model of HBV polymerase using HIV-1 reverse transcriptase as a template[11] could be used for the construction of HBV polymerase·L-FMAU-TP complex. Therefore, the HBV polymerase domain was modeled by the composer module in Sybyl, version 6.7 (Tripos, Inc.) using the crystal structure of HIV-1 RT (PDB code 1RTD).[12] The two Mg^{2+} ions, thymidine triphosphate and template-primer duplex were located at the same position as the HIV-1 RT·DNA· dNTP complex structure (1RTD). The stability of the modeled HBV polymerase·DNA·thymidine triphosphate complex was confirmed by performing molecular mechanics energy minimization and molecular dynamics simulation by using the molecular graphics and simulation program MacroModel, version 7.0 (Schrödinger, Inc.). The complex was minimized until there was no significant movement in atomic coordinates using MMFF94s force field in the presence of GB/SA continuum water model before performing molecular dynamics simulations. A conjugate gradient, Polak-Ribiere 1st derivative method was used for energy minimization. Molecular dynamics simulations on HBV polymerase· DNA·L-FMAU-TP was performed with MMFF94s in the presence of GB/SA continuum water model on a Silicon Graphics Octane2 workstation running the IRIX 6.5 operating system by heating from 0 to 300 K over 5 ps and equilibrating at 300 K for an additional 10 ps. Production dynamics simulations were carried out for 500 ps with a step size of 1.5 fs at 300 K. A shake algorithm was used to constrain covalent bonds to hydrogen atoms. For simulation of the HBV polymerase·L-FMAU-TP complex, the residues further away than 15 Å from the active site were not considered and the residues from 6 to 15 Å were constrained by harmonic constraints. Only residues inside 6 Å sphere from the L-FMAU-TP were allowed to move freely.

The Monte Carlo conformational search of L-FMAU was performed in 5000 steps, in the presence of GB/SA water model using MM3 force field.

Results and Discussion

As was pointed out by Das et al.,[11] there were several interesting structural differences at the active sites between the template enzyme, HIV-1 reverse transcriptase (RT) and the final model of HBV polymerase. Among those differences, the most outstanding one on which we focused was Met519 in HBV polymerase which corresponds to Gln151 in RT (Fig. 1). In RT, Gln151 is tightly bound to the neighboring residues such as Arg72 and Lys73 by hydrogen bonding and participates in the formation of the '3′-OH pocket'. Since this residue is directly involved in the stabilization of the bound nucleoside triphosphate, Q151M mutation causes multidrug resistance in RT.[13] Therefore, of great structural interest was the role of Met519 at the active site of HBV polymerase (Fig. 1).

The crystal structure of L-FMAU was obtained from the Cambridge Structural Database (Fig. 2a). In conformational point of view, L-FMAU is very characteristic since the gauche effects caused by the two electron withdrawing groups at the 2′ (F) and 3′ (OH) positions (Fig. 2b) shift the equilibrium to the south (2′-*endo*) conformation (Fig. 2c).

On the other hand, the ternary complex of RT, which was used as a template for the construction of the homology model of HBV polymerase, showed that although the DNA appears to be predominantly in the B-form, the base pairs close to the polymerase active site have an A-like conformation with a widened minor groove.[12] It is important that in B-DNA the 2′-deoxysugars prefer a distinct south conformation, whereas in A-DNA the 2′-deoxysugars require puckering in the antipodal north conformation,[14] which disfavors the location of 2′-endo L-nucleoside at the active site of the

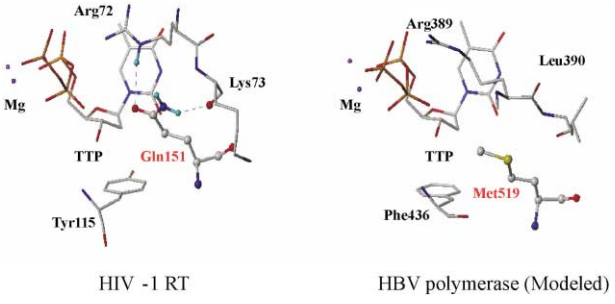

Figure 1. Comparison of the active site residues between the template (HIV-1 RT) and the modeled HBV polymerase.

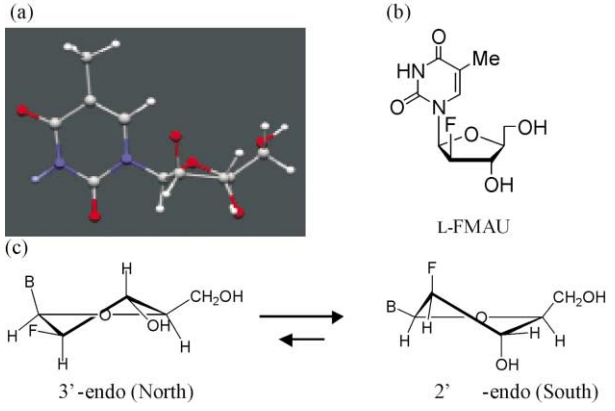

Figure 2. Structure of L-FMAU: (a) X-ray structure of L-FMAU; (b) L-FMAU has two electron withdrawing groups at the 2′ and 3′ positions; (c) Gauche effect favors 2′-*endo* (south) conformation.

polymerase. Docking the L-FMAU-TP at the active site of HBV polymerase was challenging because of the steric hindrance of the sugar moiety of L-FMAU with the 3′-end of the primer strand. Additionally, the 3′-OH group of L-FMAU was found to be in too close contact to the aromatic ring of Phe436 (Fig. 3a).

Therefore, the RT-like active site conformation may not be able to accommodate L-FMAU-TP. As the HBV polymerase has Met519, which is not involved in the specific interaction with the active site residues, the active site of HBV polymerase is not as tight as that of RT. Therefore, the conformational flexibility of HBV polymerase provided by Met519 might generate a favorable conformation to accommodate L-FMAU-TP (Fig. 3b). The conformational space of the active site of HBV polymerase was investigated by simulating the complex by molecular dynamics to give a stable ternary complex which was free of steric hindrance among the 3′-end of the primer strand, L-FMAU-TP and the aromatic ring of Phe436 (Fig. 3). The conformational adjustment initiated at Met519 propagated to the nearby Phe436 to give enough space for L-FMAU-TP (Fig. 3b) to undergo the conformational change from 2′-endo to 3′-*endo* (Fig. 4). As a result, the sugar moieties of L-FMAU-TP and 3′-terminal nucleoside of the primer strand could acquire enough space between each other (Figs. 4 and 5).

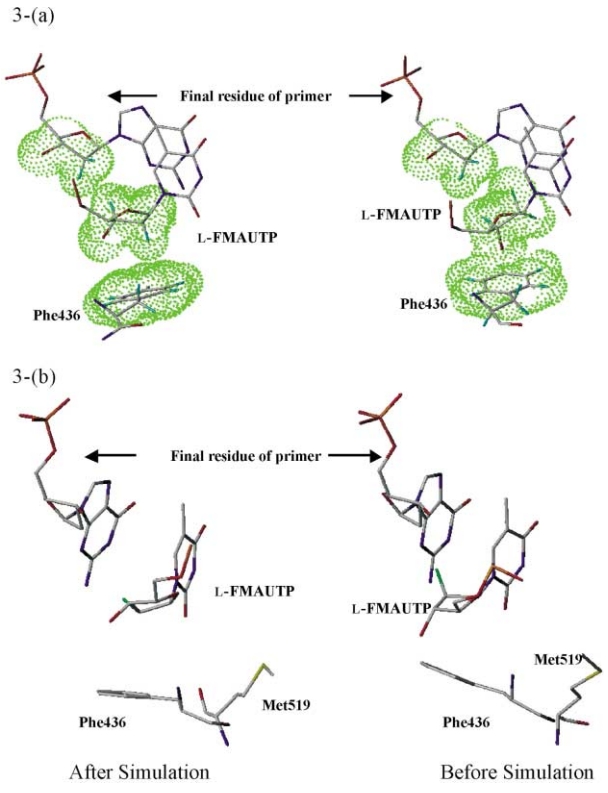

Figure 3. Before (right) and after (left) molecular dynamics simulation of HBV polymerase L-FMAU-TP complex: (a) the steric hindrance among the final primer residue, L-FMAU-TP and Phe436 is relieved after simulation; (b) the conformational change in Phe436 induced by Met519 provides enough space for L-FMAU-TP to adjust its conformation.

Even though the flexibility of the HBV polymerase gave a different active site conformation which could accommodate L-FMAU-TP, its binding should take place through a conformational change in its own sugar ring. Since the 2′-*endo* conformation of FMAU is found in its X-ray structure and believed to be the most stable, the conformational change in L-FMAU-TP can be an unfavorable process with a loss of binding energy. The relative stabilities of 2′-*endo* and 3′-*endo* conformations of L-FMAU were compared by a Monte Carlo conformational search, which found the 2′-endo conformation as a global minimum (−120.93 kcal/mol) with 45 hits. However, the 3′-endo conformer was found as stable as the global minimum conformer (−120.86 kcal/mol) of 2′-endo with 37 hits, which suggests that L-FMAU-TP can adjust its conformation to bind to the active site of HBV polymerase without significant loss of energy.

On the other hand, the conformational change in L-FMAU-TP resulted in an interesting change in the polymerization geometry of the growing viral DNA chain (Fig. 6). Since this polymerization should take place by the S_N2-type attack of the 3′-OH group at the 3′-end of the primer strand on the α-phosphorous atom of the nucleoside triphosphate, the distance and relative

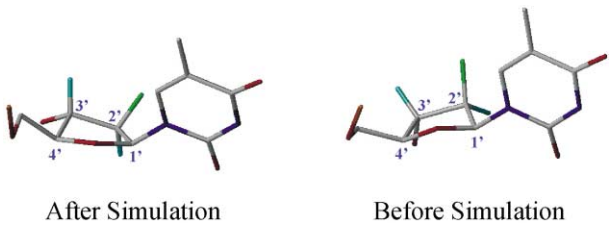

Figure 4. Conformational change of L-FMAU-TP from 2′-*endo* (right) to 3′-*endo* (left) after molecular dynamics simulation.

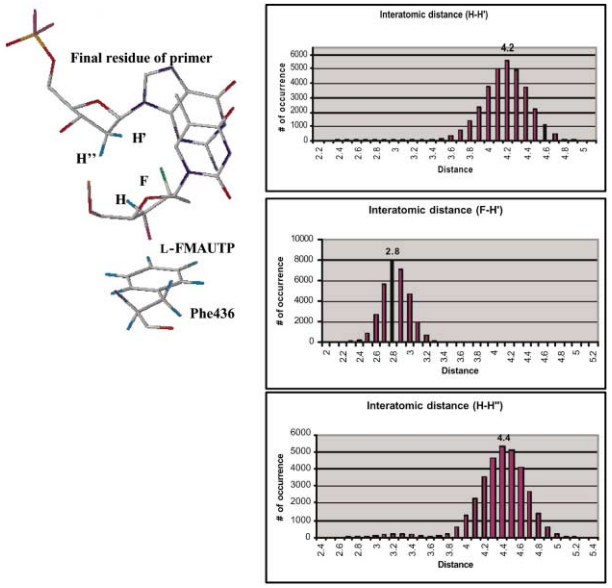

Figure 5. The monitored interatomic distances (H–H″, H–H′ and H′–F) shows that the conformational change in L-FMAU-TP provided enough space between the final primer residue and L-FMAU-TP. Among the triphosphate part of L-FMAU-TP, only α-phosphorous atom is shown for clarity.

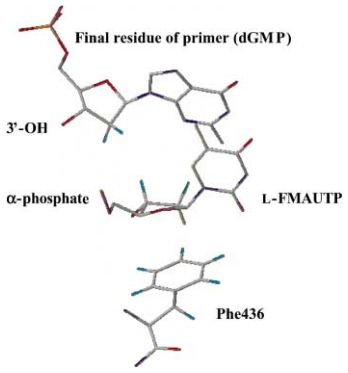

Figure 6. The conformational change in L-FMAU-TP separates its α-phosphorous atom from the 3′-OH group in the final primer residue (dGMP), which deforms the normal polymerization geometry (3.5 Å) for the viral chain elongation. Among the triphosphate part of L-FMAU-TP, only α-phosphorous atom was shown for clarity.

orientation between the two atoms are critical. As can be found in many X-ray structures of the protein including the DNA duplex and the nucleoside triphosphate substrate, the optimum distance between the nucleophile (3′-OH at the primer end) and the reaction center (α-phosphorous of the nucleoside triphosphate) is around 3.5 Å.[12] In L-FMAU-TP binding to the active site of HBV polymerase, however, as the sugar moiety of L-FMAU-TP separates from the 3′-end of the primer strand by the conformational change, its α-phosphate atom also moves out from the 3′-OH group of the primer end by more than 2 Å (5.7 Å) (Fig. 6). Therefore, the incorporation of L-FMAU-TP to the growing viral DNA chain to act as a chain terminator would not take place easily because of this deformed polymerization geometry, which may be the reason that L-FMAU is not incorporated to the HBV DNA.

In summary, because of the unique conformation of L-FMAU, the binding of its triphosphate to the active site of HBV polymerase should take place with conformational changes at the enzyme active site as well as the L-FMAU-TP itself. During this conformational change, however, the nucleophile and the reaction center for the S_N2-type DNA chain elongation separate from each other to give a deformed polymerization geometry. Taken together, by the molecular dynamics simulation study of HBV-polymerase· DNA·L-FMAU-TP complex, we have demonstrated that L-FMAU-TP can act as a competitive inhibitor by binding to the active site of HBV polymerase, but not act as a substrate because the binding of L-FMAU-TP to the active site of HBV polymerase cannot take place without the conformational adjustment, which prevents L-FMAU-TP from being incorporated into the growing viral DNA chain. This result may suggest that L-FMAU-TP occupies the catalytic site of the polymerase, thereby inhibits the priming of the HBV DNA chain elongation.

Acknowledgements

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