Molecular Modeling Approach to Understanding the Mode of Action of L-Nucleosides as Antiviral Agents

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A series of unnatural L-nucleosides such as 3TC, FTC and L-FMAU have been found to be potent antiviral agents. The mode of action of L-nucleosides has been found to be similar to that of D-nucleosides as antiviral agents, despite their unnatural stereochemistry, that is, nucleotide formation by kinases followed by interaction with the reverse transcriptase (RT) of HIV or DNA polymerase. To date, the mode of action of nucleoside inhibitors at the molecular level with respect to the active conformations of the 5'-triphosphates as well as the interaction with the RT is not known. Recently, the X-ray crystal structure of the RT-DNA-dTTP catalytic complex has been reported. Computer modeling has been performed for several pairs of D- and L-nucleoside inhibitors using the HIV-1 RT model and crystal coordinate data from a subset of the protein surrounding the deoxynucleoside triphosphate (dNTP) binding pocket region. Results from our modeling studies of D-/L-zidovudine, D-/L-3TC, D-/L-dideoxycytosine triphosphates, dTTP and dCTP show that their binding energies correlate with the reported 50% effective concentrations. Modeling results are also discussed with respect to favorable conformations of each inhibitor at the dNTP site in the polymerization process. Additionally, the clinically important M184V mutation, which confers resistance against 3TC and FTC, was studied with our modeling system. The binding energy patterns of nucleoside inhibitors at the M184V mutation site correlate with the reported antiviral data.

Antiretroviral therapy for the treatment of human immunodeficiency virus type 1 (HIV-1) infection has proven effective in extending the life and enhancing the quality of life of patients with AIDS (25). Thus far, six nucleoside reverse transcriptase (RT) inhibitors (NRTIs), four protease inhibitors, and three non-NRTIs have been approved by the Food and Drug Administration. In particular, NRTIs continue to be the mainstay of antiretroviral therapy (24, 31). For example, triple-combination therapy, consisting of zidovudine (AZT) (3'-azido-3'-deoxythymidine) (9, 17, 18, 21, 28), 3TC [(–)-β-L-2',3'-dideoxy-3'-thiacytidine] (7, 33, 34), and a protease inhibitor, is being used as the primary regimen for AIDS treatment (11, 15). Therefore, a complete understanding of the mechanism of action of NRTIs at the molecular level continues to be an important scientific objective for design and development of more effective and less toxic agents.

The NRTIs bear structural features common to 2',3'-dideoxynucleosides, and the majority of the approved drugs have the natural D configurations: AZT, ddC (2',3'-dideoxycytidine) (1, 5, 46), ddI (2',3'-dideoxyinosine) (8, 12, 29, 47), d4T (2',3'-didehydro-2',3'-dideoxythymidine) (16, 22), and abacavir (1592U89; succinate) (10, 38). Since the discovery of 3TC, however, a number of nucleosides with the unnatural L configuration have emerged as potent antiviral agents. Both 3TC and FTC [(–)-β-L-2',3'-dideoxy-5-fluoro-3'-thiacytidine] show potent antiviral activity against HIV and hepatitis B virus, with favorable pharmacokinetic and toxicity profiles (20, 43). Therefore, structural features and conformational preferences of the D and L enantiomers, as well as their interactions with

the target enzymes, have been the critical issue to be studied (4, 26, 27, 39, 40, 43).

The activation of nucleoside RT inhibitors involves two major events: phosphorylation by kinases and the interaction of the deoxynucleoside triphosphate (dNTP) with the RT (14, 30, 35). The antiviral activity of 2',3'-dideoxynucleosides is dependent on their phosphorylation by cellular kinases in the cytoplasm to the corresponding 5'-triphosphates. These triphosphates compete with the corresponding endogenous nucleoside triphosphates at the catalytic site of the HIV-1 RT, and also, upon incorporation into the nascent DNA strand the nucleotides act as chain terminators of the DNA elongation. The initial phosphorylation of nucleosides requires several cellular kinases, such as thymidine kinase, deoxycytidine kinase, and adenosine kinase, and the activities of these kinases depend on the nature of the heterocyclic base as well as the structure and stereochemistry of the carbohydrate moiety (36).

However, as three-dimensional structures of these kinases have not yet been determined, it is difficult to envision how the initial phosphorylation is carried out for unnatural nucleosides, such as L-nucleosides, without compromising the stereochemical requirements of the enzymes and/or the nucleosides. Furthermore, the active conformation of the 5'-triphosphates at the site of the RT is not well understood. Recently, Huang et al. reported the X-ray structure of the covalently trapped catalytic complex of the HIV-1 RT with dTTP and the primer-template duplex (19).

In attempts to understand the molecular-level interaction of D- and L-nucleoside triphosphates at the catalytic site of the HIV-1 RT, we performed molecular modeling studies based on the X-ray structure of RT-DNA-dTTP (19). On the basis of these studies, we were able to explain how D- and L-nucleoside triphosphates interact with the active site of the HIV-1 RT

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FIG. 1. Structures of the nucleosides investigated.

(Fig. 1). Furthermore, we were able to correlate the binding energy of the preferred conformation of the triphosphates at the RT level to the reported antiviral data. The ability to correlate the anti-HIV activity with the molecular structure as well as the stereochemistry of nucleosides and nucleotides is of considerable significance, not only for understanding the molecular mechanism but also for providing potentially predictive information for future drug design.

MATERIALS AND METHODS

Criteria defining the model enzyme site. In order to model the catalytic complexes of the polymerase active site of the HIV-1 RT, a modified enzyme site surrounding the dNTP pocket was constructed from the coordinates of RT-DNA-dTTP (19). The enzyme site consists of amino acid residues 51 to 242 from the p66 domain, double-stranded DNA template-primer at a 7:4 ratio, two magnesium ions, and dNTP. Magnesium ions are important to provide the right orientation of dNTP with respect to the DNA duplex as well as the enzyme, since these metals interact with several amino acid residues (including three aspartic acids) and the pro- R_p oxygens on the α - and β -phosphates of the dNTP at the active site.

We have investigated several NRTIs for two variables. First, we calculated the binding energies of each enantiomer with respect to the RT model, including the biologically inactive nucleoside, such as L-AZT. Subsequently, we compared the calculated relative binding energy from energy minimizations to the reported in vitro biological data. Each relative binding energy was compared to the binding energy of dTTP or dCTP at the enzyme site, reconstructed from the catalytic complex of the HIV-1 RT (Table 1). The interaction between dNTP and the active site was also analyzed after energy minimization, including the sugar conformation of the dNTP. Second, in order to understand the molecular-level interaction of NRTIs with the mutated RT conferring resistance to 3TC, the M184V mutation was also constructed as the wild-type enzyme site, the relative binding energies of several dNTPs were calculated, and the results were analyzed (Table 2) (37). The patterns of viral resistance have been well studied in clinical

isolates from early monotherapy trials as well as from more recent drug combination trials. Certain mutations, such as M184V, are sufficient by themselves for high-level drug resistance; other mutations appear to reinforce the effects of the primary mutation. The M184V mutant, which appears rapidly in monotherapy with 3TC or FTC, exhibits some cross-resistance to ddI and ddC (37). Therefore, in our study we studied the triphosphates of 3TC, ddC, and AZT along with the M184V mutant model.

dNTPs. We investigated the 5'-triphosphates of D and L pairs of nucleosides for two main sugar puckerings, such as north (3'-endo, 2'-exo) and south (2'-endo, 3'-exo) conformations according to the pseudorotational angle of the ribose ring to determine the favored conformation at the catalytic site of the HIV-1 RT. The interaction of the triphosphate moiety of each dNTP with the RT allows the ribose ring and heterocyclic moieties to bind to the active site. Due to the pivotal role played by the triphosphate moiety in the orientation of the dNTP within the active site, the conformation displayed by dTTP at the catalytic site of RT-DNA-dTTP (19) was used. The heterocyclic base moiety was then situated in such an orientation that it could be paired with its complementary base in the template strand.

Method. Molecular modeling was conducted with Sybyl (Tripos Associates; version 6.5), with all minimizations performed using the Kollman-All Atom Force Field (44) within the Biopolymer module on a Silicon Graphics Indigo 2 graphic workstation or an Octane workstation. The modeling calculations were based on the X-ray coordinates of the HIV-1 RT complexed with dTTP and the primer-template duplex reported by Huang et al. (19). A modified site, surrounding the nucleoside inhibitor binding pocket (dNTP pocket), was constructed from the enzyme coordinates and contained two magnesium ions, a truncated p66 subdomain, and the DNA duplex. Hydrogen atoms were added to the enzyme, the duplex, and the nucleoside triphosphates. The construction of nucleoside triphosphates of interest was based on the X-ray coordinates in the Cambridge Crystallographic Database for AZT (2), FTC (41), and ddC (3); the X-ray structure of dTTP-DNA-HIV-1 RT (19); and conformational analysis using the Grid search. Kollman-All Atom charges were loaded for the enzyme and two Mg ions, and Gasteiger-Hückel charges were also loaded for dTTP, other nucleoside inhibitors, and the duplex. The catalytic site and the inhibitors were minimized until the energy change from one iteration to the next was less than 0.05 kcal/mol. This cutoff energy was used consistently throughout the optimizations. Parameterizations for the Mg ions at the active site were carried out for the Kollman-All Atom Force Field. For the 3TC-resistant mutant, the M184V mutant enzyme site was constructed to be the same size as the wild-type site by point mutation of the amino acid residue Met 184 to Val 184, followed by the application of the side chain conformation fix function from the Biopolymer module. The inhibitors were merged to the active site, and the resulting complexes were minimized. The binding affinities of the examined structures for the HIV-1 RT were predicted by the binding energy differences between the inhibitor-enzyme complexes and the dTTP-RT or dCTP-RT complex in the energy-minimized states. The binding energy and relative binding energy obtained are listed along with the reported in vitro data in Tables 1 and 2.

RESULTS AND DISCUSSION

D- and L-nucleotides in a wild-type enzyme active-site model. The binding energies of various nucleoside inhibitors with re-

TABLE 1. Correlation between binding energies of D- and L-nucleoside triphosphates at sites constructed from crystal structure coordinates and EC_{50} ^a

dNTP	E_b (kcal/mol)	E_r (kcal/mol)	Sugar conformation	EC_{50} (μ M) ^b	Cell line
dTTP	268.2		3'-endo		
dCTP	285.2		3'-endo		
AZTTP	338.6	70.4	3'-endo	0.004	PBM (33)
L-AZTTP	93.5	-174.7		>100	MT-4 (44)
(+)-D-BCHTP	343.0	57.8		0.21	PBM (6)
3TCPT	355.6	70.4		0.002	PBM (32)
ddCTP	306.8	21.6		1.5	CEM (22)
L-ddCTP	295.1	9.9		5.0	CEM (22)

^a EC_{50} , 50% effective concentration; E_b , binding energy, calculated as energy of inhibitor + energy of wild-type enzyme site - energy of enzyme-inhibitor complex; E_r , relative E_b , calculated as E_b of nucleoside triphosphate - E_b of dTTP or dCTP.

^b In vitro data for the nucleoside analogue.

FIG. 2. (A) AZTTP complexed with the enzyme site. The active site of the enzyme is represented by a Connolly surface, and the inhibitor and the DNA are shown as capped sticks (colored by atom). Mg^{2+} ions are magenta. (B) Comparison of the RT-DNA-L-AZTTP complex (magenta) with the RT-DNA-AZTTP complex (yellow). (C) 3CTCP complexed with the enzyme site. (D) (+)-D-BCHTP complexed with the enzyme site.

spect to that of dTTP or dCTP show interesting correlations with the reported biological data (Table 1).

AZTTP (AZT-5'-triphosphate), which bears an additional chiral center at the 3' position of the ribose moiety of the 2',3'-dideoxynucleosides, fits well in our model in terms of the energy and molecular-level interaction (Table 1 and Fig. 2A). In the X-ray structure of the catalytic complex of HIV-1 RT-DNA-dTTP, the 3'-OH group of the dTTP projects into a small pocket formed by the side chains of Asp 113, Tyr 115, Phe 116, and Gln 151 as well as the peptide backbone between positions 113 and 115. The 3'-OH pocket can accommodate several water molecules other than the 3'-OH group, and it has room for a larger group, e.g., an azido moiety of AZTTP (19). After energy minimization of AZTTP with the enzyme site model, the 3'-azido moiety of AZTTP fitted nicely in the

pocket, along with the hydrogen bonding to the NH group of Tyr 115, which may contribute to the enhanced binding affinity of this inhibitor. AZTTP is positioned in such a way that it can maintain Watson-Crick base pairing between A and T. Also, we have found that only the 3'-endo (north) conformation of AZTTP fits into the active site of the HIV-1 RT, while it has been known that 2'-endo (south) conformation of AZT is favored at the kinase phosphorylation level (2, 39, 40). The result suggests that AZT by its flexible nature may adopt the south conformation required for the initial phosphorylation and subsequently switch to a north conformation in the triphosphate state for better interaction with the active site of the HIV-1 RT. Our observation is supported by the earlier study in which the inhibition of HIV-1 RT occurred almost exclusively with the conformationally locked carbocyclic AZT

TABLE 2. Comparison of binding energies of selected nucleoside triphosphates at the M184V mutant enzyme site with EC₅₀^a

dNTP	E_b (kcal/mol)	E_r (kcal/mol)	EC ₅₀ (μM) ^b	Cell line
dTTP	322.0			
dCTP	348.1			
3TCTP	-1,970.7	-2,318.8	>500	MT-4 (36)
AZTTP	371.7	49.7	0.03	MT-4 (36)
ddCTP	354.0	5.9	0.77	MT-4 (36)

^a EC₅₀, 50% effective concentration; E_b , binding energy for the M184V mutant enzyme site, calculated as energy of inhibitor + energy of M184V mutant site - energy of M184V mutant site-inhibitor complex; E_r , relative E_b , calculated as E_b of nucleoside triphosphate - E_b of dTTP or dCTP.

^b In vitro data for the nucleoside analogue.

5'-triphosphate analogue in the north conformation (₂E), which was kinetically indistinguishable from the inhibition produced by the AZT 5'-triphosphate, while the south (₃E) conformer did not inhibit the RT (26).

In the case of L-AZTTP, the binding affinity seems much lower than that of the natural substrate, dTTP or AZTTP (Table 1). It appears that its L configuration and 3'-azide group do not allow L-AZTTP to bind at the active site (especially the 3'-OH pocket) in such a way that Mg-phosphate coordination as well as pairing with the duplex are in order (Fig. 2B). One extra stereocenter at the 3' position of 2',3'-dideoxynucleosides, such as AZT, appears to cause significant differences in stereochemical interactions at the polymerase level. However, it is not known whether L-AZT would be recognized by the host kinases.

In contrast to AZTTP, D and L pairs of 2',3'-dideoxynucleoside triphosphates, such as 3TCTP and ddCTP, have only two chiral centers, which seem to be well tolerated at the RT level. The mode of action of L-nucleosides has been found to be similar to that of D-nucleosides as antiviral agents; that is, it follows intracellular phosphorylation to the 5'-triphosphate, which inhibits the polymerase and/or serves as the substrate for the viral enzyme, resulting in chain termination of the viral DNA (34). When the base moiety and the 5'-hydroxymethyl group are used as reference points, the furanose rings in each pair of the D- and L-nucleosides are sterically similar even though there is no superimposition of atom upon atom. This stereochemical relationship is also applicable for the triphosphates at the RT level. The differences in antiviral activity between D and L isomers seem to derive mainly from the different substrate specificities of other cellular enzymes, such as deoxycytidine kinase, thymidine kinase, or deaminase. The conformational preference of the triphosphates was not found in the case of 3TC and ddC, since any conformers could fit into the active site, including the 3'-OH pocket, and this pocket could be filled by water molecules. Based on these findings, we may conclude that only 3'-substituted 2',3'-dideoxynucleoside triphosphates in the D configuration may effectively bind to the active site of the RT, while in the case of 3'-unsubstituted 2',3'-dideoxynucleosides both the D- and L-nucleosides can be effectively bound to the enzyme. These results are supported by the fact that a number of pairs of 2',3'-dideoxynucleosides and 2',3'-unsaturated nucleosides in D and L configurations exhibit significant antiviral activity. The differences in the antiviral activities and toxicity profiles may also be due to other

factors such as drug penetration as well as anabolic and metabolic processes effected by initial kinases.

The patterns of the binding of two pairs of 2',3'-dideoxynucleosides with the enzyme active site are shown in Fig. 2C and D and 3A and B. The results from our modeling study suggest that the mode of the binding of 3TCTP and L-ddCTP to the dNTP pocket and the DNA duplex in the RT model appears to be similar to that of the natural D nucleotide, except that their β-L configurations cause the sugar portion to be shifted toward the side chain of Met 184 (Fig. 2C and 3B). Furthermore, the 3'-sulfur atom of the oxathiolane ring of 3TCTP may play an important role in enhancing the binding affinity for the HIV-1 RT by effecting additional nonbonding interaction with the side chain of Met 184, in comparison to those of D/L-ddCTP and dCTP. This property of 3TC appears to offset its unnatural L configuration, and the RT may be able to incorporate its triphosphate as the substrate. Related aspects are discussed in a kinetic study on the incorporation of 3TCTP and (+)-D-BCHTP by the HIV-1 RT as well as molecular modeling reported by Feng and Anderson (13). The data suggested that perturbations on the ribose ring of cytidine analogues (C to S) decrease the rate and efficiency of incorporation but enhance the binding affinity (13).

M184V mutant model. The binding energy patterns of nucleotides at the M184V mutant site correlate with the reported antiviral data (Table 2). In the wild-type HIV-1 RT, the amino acid residue Met 184 is part of the highly conserved YMDD motif, which contains two of the three essential aspartic acids (Asp 110, Asp 185, and Asp 186) that define the polymerase active site. The M184I/V point mutation is sufficient to cause resistance to 3TC with some cross-resistance to ddI and ddC. M184I/V is in the neighborhood of the incoming nucleotide. This mutation is likely to affect the position, stability, and reactivity of the bound analogue (19). The orientation of the incoming dNTP is affected by a set of protein side chain contacts as well as by the two metal (magnesium) ions. Small changes in the contacting residues could markedly influence the rate of nucleotide incorporation. The methionine side chain attaches to the sugar and the base of the 3'-nucleotide in the primer. Huang et al. suggested that the introduction of a β-branched side chain, as in the case of isoleucine and valine, may facilitate contact with the dNTP sugar ring (19). In particular, 3TCTP incorporation into the active site of the M184V mutant may be severely hindered by the M184V substitution due to 3TCTP's 1,3-oxathiolane ring and β-L configuration, in contrast to that of the natural dNTP.

Energy minimization of 3TCTP at the M184V mutant site showed that 3TCTP is situated in such a way that the steric conflict with the side chain of Val 184 is avoided, resulting in instability of overall conformations of the complex, including the primer-template duplex. As illustrated in Fig. 3C, 3TCTP at the M184V mutant site (magenta color) is shifted away from Val 184, and subsequently the conformation of the DNA duplex is relatively distorted compared to the wild-type catalytic complex (yellow in Fig. 3C). This phenomenon can also be seen in the significant decrease in 3TCTP's relative binding energy change in the M184V mutant site model (70.4 versus -2,318.8 [Tables 1 and 2, respectively]), which correlates with the loss of anti-HIV-1 activity. Our results are also supported by the recent report by Sarafinas et al. (32) in which steric

FIG. 3. (A) ddCTP complexed with the enzyme site. The active site of the enzyme is represented by a Connolly surface, and the inhibitor and the DNA are shown as capped sticks (colored by atom). Mg^{2+} ions are magenta. (B) L-ddCTP complexed with the enzyme site. (C) Comparison of the M184V RT-DNA-3TCTP complex (magenta) with the wild-type RT-DNA-3TCTP complex (yellow).

significantly affected by the substitution at position 184, probably due to the fact that the β -D-sugar portion of AZTTP is distant from the amino acid residue Val 184 in comparison to that of 3TCTP. These results may explain the ability of the M184V mutant RT to recognize and incorporate other dNTPs as substrates while rejecting 3TC or FTC triphosphates. It is also expected that other dideoxynucleosides with the β -L configuration might be cross-resistant to the M184V mutant to a certain extent.

In summary, in attempts to understand the mode of action of L-nucleosides, we have studied the binding of several pairs of D- and L-nucleoside triphosphates at the HIV-1 RT catalytic site by molecular modeling based on the X-ray structure of HIV-1 RT-dTTP-DNA. The results indicate that the calculated relative binding energies of the triphosphates generally correlate with their in vitro anti-HIV-1 activities. Furthermore, from these studies we could explain, at least partially, the effect of the M184V mutation on antiviral efficacy in vitro. These observations may lead to the development of a predictive tool for designing new anti-HIV agents.

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hindrance between 3TCTP and Ile 184 of the M184I mutant RT could shift the binding positions of inhibitors. On the basis of the crystal structure of M184I RT-DNA and a modeling study of M184I RT-DNA-3TCTP, it was suggested that resistance to 3TC may be the result of perturbed stereochemistry of 3TCTP binding in the catalytic complex, leading to an abortive transition state for the viral polymerization process (32).

ddCTP was also modeled in this system, and the result (Table 2) suggested that the binding affinity of ddCTP seems to be moderately decreased (21.6 versus 5.9); however, in vitro antiviral data (50% effective concentration, 0.77 μ M) in Table 2 do not clearly indicate that ddC is cross-resistant to the M184V mutant. AZT, which exhibits antiviral activity against the 3TC-resistant mutant, was also included in this modeling study (Table 2). As expected, the binding affinity of AZTTP was not

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