Defining a Molecular Mechanism of Synergy between Nucleoside and Nonnucleoside AIDS Drugs*

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Combination therapies treating human immunodeficiency virus type 1 (HIV-1) infection delay the emergence of drug-resistant virus and exhibit synergistic inhibition. This synergy is observed within the two classes of inhibitors that target the essential viral reverse transcriptase (RT): the chain-terminating nucleoside analogs (NRTIs) and the allosteric nonnucleosides (NNRTIs) that bind in a pocket distinct from the active site. A general mechanism to define the molecular basis for synergy between these two classes remains to be elucidated. Previous mechanistic studies from our laboratory (Spence, R. A., Kati, W. M., Anderson, K. S., and Johnson, K. A. (1995) Science 267, 988–993) have shown that the natural deoxynucleoside triphosphate and the NNRTI can simultaneously bind to their respective sites. This work also suggests communication between the two sites, since the inhibition of RT by NNRTIs is manifested through a remote effect on the chemical step. This interplay between the two sites offers a plausible hypothesis for understanding synergy in which binding of NNRTIs modulates the chain termination by NRTIs. The present study supports this hypothesis by illustrating that the clinically approved NNRTIs, nevirapine and efavirenz, inhibit the ATP-mediated removal of AZTTP, d4TTP, ddCTP, (-)3TCMP, (-)FTCMP, and (+)3TCMP, thereby prolonging the effectiveness of chain termination. This inhibition is mediated through an effect on both the rate of the chemical step and binding of ATP, resulting in an overall decrease in efficiency of removal. This work substantiates communication between the two binding pockets, the sustained use of combination therapy to treat HIV infection, and a molecular basis for understanding synergy.

Monotherapy treatment against HIV-1 infection can be beneficial in decreasing viral load but is ultimately rendered ineffective by the appearance of drug-selected mutations. Combination therapy, however, strives to delay the emergence of drug-selected mutations and viral resistance (1, 2). Highly active anti-retroviral therapy includes inhibitors of the HIV-1 reverse transcriptase (RT) and the protease (PR). While multiple-drug therapies comprising of RT and PR inhibitors delay resistance, cocktails of these inhibitors can also exhibit additive or synergistic inhibition of viral replication (3). Intriguingly, synergy is also observed within two classes of RT inhibitors, that is, the nucleoside RT inhibitors (NRTIs), can show synergy with the nonnucleoside RT inhibitors (NNRTIs) in the absence of PR inhibitors (4). For example, the NRTI, AZT (zidovudine), and the NNRTI, nevirapine, exhibit synergy when given in combination to cultured cells (5). This increase in potency from combination therapy has been observed among other combinations of NRTIs and NNRTIs, both in cultured cells and in patients (6–9).

These two classes of inhibitors are functionally distinct in their mode of action. The nucleoside analog family of inhibitors is prodrug nucleosides, which are transported across host cellular membranes and phosphorylated to the metabolically active nucleotides. These nucleotides serve as substrates for RT, and lacking a 3'-hydroxyl group, they serve to chain-terminate a growing DNA strand. This chain termination and their competition with natural dNTPs yields their therapeutic value (10). However, previous studies have demonstrated that RT can catalyze either ATP or PPi-mediated removal of chain-terminating analogs (11–13). ATP and PPi act as nucleophiles that directly attack the phosphodiester linkage to form either an ATP analog dinucleoside tetraphosphate (14) or the triphosphate of the analog, respectively, leaving behind the primer poised for incorporation once again.

Structural evidence shows that the allosteric nonnucleoside inhibitors bind to a pocket ~10 Å away from the polymerase site (15, 16), and steady-state kinetic studies suggest they are noncompetitive with respect to binding of dNTP and primer/template (17–21). Despite much published work on the modes of inhibition by NRTIs and NNRTIs, how they mechanistically display synergy observed in vivo and in vitro has largely remained unknown. It is unclear if a general mechanism exists that may account for synergy observed.

Earlier mechanistic studies from our lab and others (22, 23) have illustrated that the natural deoxynucleoside triphosphate dNTP and the NNRTI can simultaneously occupy their respective sites. Moreover, this work also suggests that communication occurs between the active and allosteric sites, since the inhibition of RT by NNRTIs is manifested through a remote effect on the chemical step. At the molecular level, transient kinetic studies have shown that nonnucleoside inhibitors change the rate-limiting step of catalysis, resulting in chemistry as the slowest step (22, 23). This interplay between the two sites offers a plausible hypothesis for understanding the mo...
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Fig. 1. NNRTIs inhibit removal of NRTIs. A, polyacrylamide gels showing single turnover removal of AZTMP, d4TMP, ddCMP, (−)3TCMP, (−)FTCMP, and (+)3TCMP in the absence of NNRTI, with 10 μM nevirapine, or with 10 μM efavirenz (3 mM ATP, 250 mM RTWT, and 50 nM of primer/template). The time course for AZTMP and d4TMP removal is 0, 5, 20, 40, 90, and 180 min; time course for all others is 0, 30, 60, 90, 180, and 360 min. Note in most cases RT removed analog and the next dNMP. B, plot of AZTMP removal in the absence (●) or presence (○) of 3 μM nevirapine, with rates of 0.00097 and 0.00051 s⁻¹, respectively. B, dependence of removal rate on ATP concentrations fit to a hyperbolic curve, yielding $K_{\text{cat}}$ values of 0.0012 ± 0.0001 and 0.0007 ± 0.00004 s⁻¹ and $K_p$ values for ATP of 0.83 ± 0.25 and 1.3 ± 0.2 mM in the absence (●) or presence (○) of 3 μM nevirapine, respectively. The addition of nevirapine decreases the efficiency value from 0.0014 ± 0.0004 to 0.00054 ± 0.00009 s⁻¹.

Fig. 2. Removal of AZTMP in the presence of nevirapine. A, single turnover removal assay showing ATP-mediated removal (3 mM ATP) of AZTMP in the absence (●) or presence (○) of 3 μM nevirapine, with rates of 0.00097 and 0.00051 s⁻¹, respectively. B, dependence of removal rate on ATP concentrations fit to a hyperbolic curve, yielding $K_{\text{cat}}$ values of 0.0012 ± 0.0001 and 0.0007 ± 0.00004 s⁻¹ and $K_p$ values for ATP of 0.83 ± 0.25 and 1.3 ± 0.2 mM in the absence (●) or presence (○) of 3 μM nevirapine, respectively. The addition of nevirapine decreases the efficiency value from 0.0014 ± 0.0004 to 0.00054 ± 0.00009 s⁻¹.

EXPERIMENTAL PROCEDURES

Expression and Purification of HIV-1 RT—The RTWT clone was generously provided by Stephen Hughes, Paul Boyer, and Andrea Ferris (Frederick Cancer Research and Development Center, Frederick, MD). N-terminal histidine-tagged heterodimeric p66/p51 transcriptase was purified as described previously (26).

Materials—AZTTP was purchased from Moravek Biochemicals. d4T was purchased from Sigma, and phosphorylation to the triphosphate of (−)3TC, (−)FTC, and (−)3TC were kindly provided by Dr. R. F. Schinazi (Emory University, Atlanta, GA). Efavirenz was purchased from Toronto Research Chemicals Inc., North York, Ontario. NNRTIs were diluted in dimethyl sulfoxide. ATP was purchased from Sigma and treated with thermostable pyrophosphatase (Roche Applied Science) to degrade any contaminating pyrophosphate.

Labeling and Annealing of Oligonucleotides—Primers and templates were synthesized at the Kec Facility at Yale University and purified with 20% polyacrylamide denaturing gel electrophoresis. The sequences of primers and templates used in this study are: D23A (5'-TCA GGT CCC TGT TCC GGC GCC AC-3'), D23B (5'-GCG TCG CAG CGG TCC AAC CA-3'), D22A (5'-TCA GGT CCC TGT TCC GGC GCC AC-3'), D22B (5'-GTC TCA GGT CCC TGT TCC GGC GCC A-3'), D36 (5'-CTA GTC GGT CCC CCA CCT TCA CCA C-3'), D36 (5'-GTC TCA GGT CCC CCA CCT TCA CCA C-3'), and D45 (5'-GTC TCA GGT CCC CCA CCT TCA CCA C-3'), D23A-AZTMP, D22A-d4TMP, D22B-(−)3TCMP, D22B-(−)FTCMP, and D23B-(−)3TCMP primers were made with previously reported methods (28). D22B-ddCMP was synthesized by the Kec Facility and purified as stated above. Primer/templates were labeled and annealed as previously described (29). Primer/templates combinations studied were D23A-AZTMP/3D36, D22A-d4TMP/D45, D22B-ddCMP/D36, D23B-

Recent work showing that synergy may be mediated between the nucleoside analog AZT and the nonnucleoside UC781 through a nucleotide removal pathway (24) lends support for the hypothesis. In this study, it was proposed that UC781 resensitizes AZT-resistant virus to AZT as a result of decreased step. This work substantiates communication between the two inhibitory pockets and a molecular basis for understanding synergy.

NNRTIs modulates the chain termination by NRTIs.

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RESULTS AND DISCUSSION

Many combinations of NRTIs and NNRTIs inhibit viral growth synergistically, including AZT and efavirenz, d4T and nevirapine, and (−3TC and efavirenz (3, 31, 32). An understanding of this phenomenon improves the quality of treatment for current patients and provides a platform for developing new therapies, but a general mechanism that accounts for this synergy is unknown.

In the context of the ability of RT to remove chain terminating nucleoside analogs in the presence of PP, or ATP (11–13,30), previous reports have suggested that NRTIs may inhibit removal of AZT monophosphate, consequently maintaining chain termination at the primer end (24, 25). The role of pyrophosphate in removal is controversial, as our laboratory and others have shown WT RT removes AZTMP with a rate higher than that of AZT-resistant RT at a physiological concentration of pyrophosphate, which argues against PP-mediated removal as a mechanism of AZT resistance (14, 30). Alternatively, AZT-resistant RT removes AZTMP more rapidly than WT RT at a physiological concentration of ATP (30). It has been established that ATP binds directly to RT at a site proximate to the active site (33) with the γ-phosphate of ATP, acting as a nucleophile, directly attacking the phosphodiester linkage between AZTMP and the primer. The products of this nucleophilic attack are the primer (free to incorporate dNTPs) and the dinucleoside tetraphosphate 3′-azido-3′-deoxythymidine(5′)-tetraphosphate(5′)-adenosine (AZTtpA). Together, these observations would suggest that ATP, and not PP, acts to mediate AZTMP removal in the context of AZT resistance. We have thus chosen to study only ATP-mediated removal in this work, examining a host of Food and Drug Administration-approved compounds to determine whether a general mechanism of synergy exists. As shown in Fig. 1A, we show that the addition of 10 μM nevirapine or efavirenz inhibits ATP-mediated removal of a wide panel of nucleoside analogs, including AZT, d4T, dC, (−3TC, (+3TC (to study an isomeric effect), and recently approved (+3FTC. These results substantiate the broad ability of RT to remove chain-terminating analogs and that there exist interactions between the polymerase active site and nonnucleoside binding pocket (22). In all cases, efavirenz inhibited removal more effectively than nevirapine and accordingly parallels the potencies observed in the clinic. The relationship between NNRTI inhibition of nucleotide removal and stereoselectivity was examined by comparing primers chain-terminated with the oxothiolane stereoisomers, (+3TCMP or (−3TCMP. In each case, the effects of NNRTI inhibition on the rates of removal for (+3TC or were comparable with that of (−3TC, showing that there is no stereochemical preference at the site of chain termination. Fig. 1B illustrates the clear inhibition of removal when either nevirapine or efavirenz is added to the assay, in that both the rate and amplitude of AZTMP removal are decreased when an NNRTI is added. It is also interesting to note that in comparing removal by wild-type RT in the absence of NNRTIs, we see that “thymidine (T)” analogs are removed more effectively than the “deoxyctydine (C)” analogs (compare time scale for d4TMP removal versus dCMP removal, 3 and 6 h, respectively.).

In considering the mode of inhibition by which NNRTIs may affect ATP-mediated removal of NRTIs, the nonnucleoside inhibitors may have a direct effect on ATP binding and/or an indirect effect on the chemical step. To further delineate this mechanism, the ATP-mediated removal of AZTMP was followed in the presence of various concentrations of ATP in the absence or presence of a fixed concentration of nevirapine. At a physiological concentration of ATP, 3 μM nevirapine inhibited removal by 50% (Fig. 2A). Using this fixed concentration of nevirapine, the rate of removal at various ATP concentrations was determined. Fig. 2B shows that the addition of 3 μM nevirapine causes both a change in the rate of chemistry of ATP-mediated removal (kcat) (0.0012 versus 0.0007 s−1) and in the Kd (dissociation constant) of ATP (0.83 versus 1.3 mM). The efficiency of removal (defined by kcat/Kd) decreases from 0.0014 to 0.00054 mM−1 s−1 (a 2.6-fold change) with the addition of nevirapine. This clearly establishes a molecular communication between the two binding pockets in which binding of an NNRTI affects the polymerase active site and perturbs the action of ATP in mediating chain termination.

Earlier studies have established that nonnucleosides affect the active site of RT and incorporation of nucleoside analogs (22). The current study adds a new dimension for the role of site communication at the enzyme level and in terms of understanding combination therapy. These data also lend further support for the suggestion that a bifunctional inhibitor combining the functionalities of a NRTI and NNRTI could bind very tightly and specifically to RT and could be effective in the treatment of AIDS. In summary, this report provides a mechanistic foundation for NRTIs and NNRTIs in combination therapy, understanding the molecular basis of inhibition and synergy, and offers new insights for designing more potent and selective anti-HIV therapies.

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