Effects of Non-nucleoside Inhibitors of Human Immunodeficiency Virus Type 1 in Cell-free Recombinant Reverse Transcriptase Assays*

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We have employed a cell-free human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) assay to study the effects of non-nucleoside inhibitors of RT (NNRTI) by directly monitoring specific HIV DNA products using a HIV-1 genome-derived template and an oligodeoxynucleotide primer. As previously shown by ourselves and others, nucleoside analog triphosphates, e.g., 3′-azido-3′-deoxythymidine triphosphate and 2′,3′-dideoxyadenosine triphosphate, could directly inhibit HIV RT RNA-dependent DNA polymerase activity by causing chain termination, as visualized in a RT reaction that yields specific DNA products. In contrast, each of two NNRTIs, nevirapine and delavirdine, directly inhibited RT activity without causing chain termination effects. We also analyzed interactions between nucleoside analogs and NNRTIs or among NNRTIs by chain elongation/dNTP incorporation and/or steady-state kinetic assays. Combinations of nevirapine with the triphosphates of either the (−)-strand of 2′,3′-dideoxy-3′-thiacytidine or 2′,3′-dideoxyadenosine yielded additive/synergistic effects on RT activity. However, only an additive effect was observed when combinations of nevirapine and 3′-azido-3′-deoxythymidine triphosphate were employed. Combinations of nevirapine and delavirdine had an antagonistic effect on the inhibition of HIV-1 RT activity.

A key step in the HIV1 life cycle is the reverse transcription of genomic RNA into double-stranded DNA mediated by the viral-encoded multifunctional enzyme reverse transcriptase (RT) (1). Two major groups of HIV-1 RT inhibitors have been studied, nucleoside analogs and a series of non-nucleoside RT inhibitors (NNRTI). The former, such as 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxyinosine, and the (−)-strand of 2′,3′-dideoxy-3′-thiacytidine are believed to block HIV-1 replication by competitively inhibiting incorporation of nucleotide substrates by RT and causing chain termination after being incorporated in phosphorylated form into elongating viral DNA (2, 3). NNRTIs are thought to act noncompetitively by binding to a hydrophobic pocket located near the polymerase catalytic site, resulting in inhibition of RT polymerase activity (4–7). Although these two classes of drugs can generally reduce viral load, at least transiently, and may improve quality of life, problems of drug intolerance and resistance have arisen after long term therapy (8–10). A series of mutations in viral RT are responsible for resistance to both nucleoside compounds and NNRTIs (11–17), although no cross-resistance has been demonstrated among these two groups of inhibitors (14, 18, 19).

Several studies have shown that recombinant viruses that contained resistance-conferring mutations for each of nucleosides and NNRTIs possessed diminished sensitivity for both groups of compounds (19). Combinations of drugs of both types have yielded synergistic results on the inhibition of HIV-1 replication in tissue culture assays (20–22). Various combinations of nucleosides and NNRTIs are currently undergoing clinical evaluation.

To better understand the mechanism of action of NNRTIs, we performed cell-free RT reactions by directly monitoring DNA products on gels and showed that each of two NNRTIs, nevirapine and delavirdine, blocked RT-mediated generation of DNA products in the absence of chain termination. We were also interested in interactions among RT, NNRTIs, and 5′-triphosphorylated nucleoside analog (ddNTP) inhibitors of RT. Our results showed that combinations of nevirapine with each of 2′,3′-dideoxyadenosine triphosphate (ddATP), the intracellular triphosphorylated active form of 2′,3′-dideoxyinosine, 2′,3′-dideoxy-3′-thiacytidine triphosphate (3TCTP), and AZT triphosphate (AZTTP) acted additively/synergistically in either chain elongation/ddNTP incorporation or steady-state kinetic assays. However, the two NNRTIs tested, nevirapine and delavirdine, were mutually antagonistic with regard to inhibition of HIV-1 RT polymerase activity. This report on the use of NNRTIs in cell-free HIV-1 RT reactions complements previous studies on ddNTP-mediated inhibition of production of specific DNA transcripts as visualized by gel analysis (23, 24).

EXPERIMENTAL PROCEDURES

Materials—Recombinant heterodimers (p66/p51) of HIV-1 RT were expressed in Escherichia coli JM109 and purified to 95% homogeneity as described (25). Poly(A) oligo(dT)12–18, ultrapure dNTPs, NTPs, and ddATP were purchased from Pharmacia Biotech Inc; AZTTP was obtained from Moravek Biochemicals (Brea, CA). 3TCTP was kindly donated by Glaxo Group Research (Greenford, UK). Nevirapine and delavirdine were generously provided by Boehringer-Ingelheim Inc. (Burlington, Canada) and Upjohn Inc. (Mississauga, Canada), respectively. [3H]dTTP, [α-32P]ddATP and [γ-32P]ATP were purchased from Du Pont NEN. Deoxyoligonucleotides were synthesized by GSD Inc.

*Supported by grants from Health Canada and the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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§This work was performed in partial fulfillment of the requirements for the Ph.D. Degree, Faculty of Graduate Studies and Research, McGill University.

The abbreviations used are: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; NNRTI, non-nucleoside RT inhibitor; AZT, 3′-azido-3′-deoxythymidine; AZTTP, AZT triphosphate; ddATP, 2′,3′-dideoxyadenosine triphosphate; 3TCTP, 2′,3′-dideoxy-3′-thiacytidine triphosphate; ddNTP, 5′-triphosphorylated nucleoside analog; PBS, primer binding sequence; dPR, deoxyxyluronucleotide primer; Cl, combination index.
Inhibition of Chain Elongation/dNTP Incorporation—We performed cell-free RT reactions that included an RNA template (HIV-PBS) containing the primer binding sequence (PBS) as well as both 5′ unique and repeat sequences of HIV-1 genomic RNA transcribed in vitro from a recombinant pHIV-PBS construct (24). Reactions were primed with a synthetic 18-mer deoxynucleotide primer (dPR), complementary to the PBS, to yield a (−)-strand strong stop DNA product of 192 nucleotides (24).

Reverse transcription reactions were performed in a volume of 20 μl (containing 50 mM Tris-HCl, pH 7.8, at 37 °C, 75 mM KCl, 10 mM MgCl₂, 250 μM of each of the four deoxynucleoside triphosphates, 50 mM HIV-PBS RNA template, 100 mM [γ-32P]ATP end-labeled dPR primer, and 42 mM p66/p51 heterodimer of wild-type HIV-1 RT) for 1 h at 37 °C in the presence or absence of RT inhibitors. Reaction products were then electrophoresed on a 5% denaturing polyacrylamide gel as described (24), and chain termination frequency and amount of full-length (−)-strand strong stop DNA product were visualized in a Molecular Image (Bio-Rad).

Steady-state Enzyme Assays—Inhibition of HIV-1 RT RNA-dependent DNA polymerase activity was measured by employing either a heteropolymeric HIV-PBS RNA template/dPR primer and each of four dNTPs as substrates or a homopolymeric poly(rA) heteropolymeric HIV-PBS RNA template/dPR primer and each of four DNA polymerase activity was measured by employing either a synthetic 18-mer deoxynucleotide primer (dPR), complementary to the PBS, to yield a (−)-strand strong stop DNA product of 192 nucleotides (24).

Reverse transcription reactions were performed in a volume of 20 μl (containing 50 mM Tris-HCl, pH 7.8, at 37 °C, 75 mM KCl, 10 mM MgCl₂, 250 μM of each of the four deoxynucleoside triphosphates, 50 mM HIV-PBS RNA template, 100 mM [γ-32P]ATP end-labeled dPR primer, and 42 mM p66/p51 heterodimer of wild-type HIV-1 RT) for 1 h at 37 °C in the presence or absence of RT inhibitors. Reaction products were then electrophoresed on a 5% denaturing polyacrylamide gel as described (24), and chain termination frequency and amount of full-length (−)-strand strong stop DNA product were visualized in a Molecular Image (Bio-Rad).

RESULTS

Comparison of RT Inhibition Patterns Caused by Nucleoside Analogs and NNRTIs—To compare differences between inhibition patterns mediated by nucleoside analogs and NNRTIs, we used a heteropolymeric template (HIV-PBS) derived from the HIV-1 genome and dPR to generate (−)-strand strong stop DNA as well as pause products that precede the formation of such material. Products were analyzed by denaturing polyacrylamide gels. Each of two nucleoside analogs, AZTTP and ddATP, caused progressively diminishing levels of chain termination with chain elongation at complementary A and U bases, respectively, in a drug concentration-dependent fashion (Fig. 1A). In contrast, neither of the NNRTIs tested, nevirapine or delavirdine, caused chain termination but rather reduced levels of full-length (−)-strand strong stop DNA product, also in a concentration-dependent manner (Fig. 1B).

HIV-1 RT pauses preferentially at certain base sequences due to dissociation from template/primer and/or stalling during chain elongation, regardless whether RNA or DNA templates are employed (24, 26). In separate experiments, neither nevirapine nor delavirdine caused alterations in pausing compared with non-drug-treated controls (Fig. 1B). Furthermore, the sites and patterns of chain termination caused by nucleoside analogs were not affected by the presence of nevirapine (Fig. 2A), suggesting that NNRTIs did not affect interactions between RT and template/primer or nucleoside analogs. In Fig. 1B, nevirapine and delavirdine were studied separately, explaining why certain bands appear higher in the gel in the case of the former drug. However, the band patterns are the same in each case. Thus, the strong termination event seen with delavirdine about halfway down the gel is equivalent to that observed somewhat higher on the gel with nevirapine. NNRTIs have been shown to block RT-mediated polymerase catalytic reactions due to generation of an inactive conformation that does not interfere with either nucleotide binding or nucleoside-induced structural change (6, 27). Hence, NNRTIs may affect RT activity without interfering with interactions of RT with template/primer and deoxynucleotide substrates/inhibitors.

Effects of Combinations of Nevirapine and Nucleoside Ana-
AZTTP, ddATP, and 3TCTP acted in a dose-dependent manner when combined with this fixed concentration of nevirapine, to further inhibit generation of (\(-\))-strand strong stop DNA product. In each case, the extent of inhibition was greater than that obtained in the presence of the NNRTI or any of the ddNTPs acting alone.

A combination index (CI) was calculated to monitor interactions among these drugs, based on the production of full-length (\(-\))-strand strong stop DNA, as described (28). Previous work by other investigators has shown that these CI determinations provide good estimates of the effects of drug combinations, with a CI of 1 indicating an additive effect and CIs of <1 and >1 indicating synergy and antagonism, respectively (28). CI values were calculated by dividing the sum of the percentages of inhibition of generation of (\(-\))-strand strong stop DNA obtained with single agents as determined by Molecular Imager by those obtained when combinations of drugs were simultaneously employed. Fig. 2A shows the results of one such experiment repeated on three separate occasions. Analysis of the intensities of (\(-\))-strand strong stop DNA (Fig. 2A, bold arrow) yielded CIs of 0.98–1.0, 0.83–0.94, and 0.75–0.84 for nevirapine when combined with various concentrations of each of AZTTP, ddATP, and 3TCTP, respectively (Table I). These values suggest that the combinations of nevirapine and 3TCTP or ddATP had low synergistic or high additive effects on inhibition of RT polymerase activity but that AZTTP only acted

![Figure 2A](image-url)

**TABLE I**

| Drug combination | CI | Comparative chain termination frequencies |
|------------------|----|-------------------------------------------|
| Nevirapine + AZTTP | 0.98–1.0 | 0.85–0.92 |
| Nevirapine + ddATP | 0.83–0.94 | 0.86–0.95 |
| Nevirapine + 3TCTP | 0.75–0.84 | 0.93–0.97 |

a Combination indices were calculated as described according to the formula:

\[
\text{CI} = \frac{\% \text{ inhibition by nevirapine}}{\% \text{ inhibition by ddNTP}}
\]

(see Figure 2A).

b Comparative chain termination frequencies in the presence of different combinations of nevirapine and ddNTP compared with chain termination in the absence of nevirapine, arbitrarily defined as 1 in each case. Nevirapine was employed at 20 nM throughout, whereas ddNTP concentrations varied as indicated in Figure 2A. Comparative chain termination frequencies were calculated by the formula:

\[
\text{CI} = \frac{\text{intensity of chain termination bands obtained with both ddNTP + Nevirapine}}{\text{intensity of chain termination bands obtained with ddNTP alone}}
\]

\[0.98–1.0\] was obtained regardless whether AZTTP concentrations (5–20 \(\mu\)M) were varied in the presence of a fixed dose of nevirapine (20 nM) or if nevirapine concentrations (10–160 nM) were varied in the presence of a fixed level of AZTTP (5 \(\mu\)M).

AZTTP, ddATP, and 3TCTP acted in a dose-dependent manner when combined with this fixed concentration of nevirapine, to further inhibit generation of (\(-\))-strand strong stop DNA product. In each case, the extent of inhibition was greater than that obtained in the presence of the NNRTI or any of the ddNTPs acting alone.

A combination index (CI) was calculated to monitor interactions among these drugs, based on the production of full-length (\(-\))-strand strong stop DNA, as described (28). Previous work by other investigators has shown that these CI determinations provide good estimates of the effects of drug combinations, with a CI of 1 indicating an additive effect and CIs of <1 and >1 indicating synergy and antagonism, respectively (28). CI values were calculated by dividing the sum of the percentages of inhibition of generation of (\(-\))-strand strong stop DNA obtained with single agents as determined by Molecular Imager by those obtained when combinations of drugs were simultaneously employed. Fig. 2A shows the results of one such experiment repeated on three separate occasions. Analysis of the intensities of (\(-\))-strand strong stop DNA (Fig. 2A, bold arrow) yielded CIs of 0.98–1.0, 0.83–0.94, and 0.75–0.84 for nevirapine when combined with various concentrations of each of AZTTP, ddATP, and 3TCTP, respectively (Table I). These values suggest that the combinations of nevirapine and 3TCTP or ddATP had low synergistic or high additive effects on inhibition of RT polymerase activity but that AZTTP only acted...
additively with this NNRTI. We also assessed the effect of varying the nevirapine concentration in this system (10–160 nM) in the presence of a fixed concentration of AZTTP (5 μM). Fig. 3 shows that the extent of inhibition of synthesis of (−)-strand strong stop DNA increased at higher drug concentrations. However, no synergy could be detected on the basis of the calculations described in Table I. This experiment was performed on each of three occasions and yielded similar results each time.

We also analyzed comparative chain termination frequencies obtained in the presence of ddNTPs plus nevirapine versus ddNTPs alone by phorphor imaging analysis of all chain termination bands (Table I). We found that more extensive chain termination occurred when nevirapine was employed together with 3TCTP than with either AZTTP or ddATP. In the presence of both nevirapine (20 μM) and ddNTPs, comparative chain termination frequencies were 0.85–0.92, 0.86–0.95, and 0.93–0.97 for various concentrations of each of AZTTP, ddATP, and 3TCTP, respectively.

In order to assess drug interactions in a different system, we selected several concentration ranges of each of nevirapine and nucleoside analog 5'-triphosphates, i.e. AZTTP, ddATP, and 3TCTP, for purposes of steady-state kinetic analysis with the heteropolymeric template (HIV-PBS) and DNA primer (dPR). The Dixon plot method was used to fit the plot of dAMP incorporation. Nevirapine showed nearly parallel lines of RT inhibition in this assay when combined with each of several different nucleoside analog triphosphates, i.e. AZTTP (Fig. 4A), ddATP (Fig. 4B), and 3TCTP (Fig. 4C). These data indicate that these combinations additively inhibit RT RNA-dependent DNA polymerase activity in this system.

Antagonistic Effects of Nevirapine and Delavirdine—To explore interactions between NNRTIs, we performed steady-state RT assays by employing poly(rA) oligo(dT) as template/primer and [3H]dTTP as substrate. Inhibition of [3H]dTMP incorporation was monitored in the presence or absence of nevirapine, delavirdine, or both. If both inhibitors cannot bind to the enzyme at the same time, i.e. their binding is mutually exclusive, the slope of 1/V (where V indicates the reaction velocity) versus the concentration of one inhibitor will be independent of the concentration of the other inhibitor. However, if both inhibitors bind simultaneously, i.e. their binding is nonexclusive, the family of lines at different fixed concentrations of the second inhibitor will intersect (29). The results of Fig. 5 show intersections of these lines. Furthermore, the inhibitory efficiency of low concentrations of nevirapine combined with high concent...
It is interesting that the CIs reported from tissue culture studies for combinations of AZT and nevirapine varied between 0.24 and 0.8 (21, 22), indicating a greater degree of synergy than that reported here for combinations of these drugs. In contrast, both 3TCTP and ddATP seem either highly additive or weakly synergistic with nevirapine in our cell elongation/dNTP incorporation system. Certain of these variations may be attributable to the different systems employed. It is also noteworthy that recombinant RT, which contains AZT resistance-conferring mutations, had similar K, values for AZTTP as wild-type enzyme (31). In contrast, K65R and L74V mutated RTs, derived from viruses resistant to ddC and 2',3'-dideoxyinosine, respectively, had increased K, values for ddCTP and ddATP with respect to their wild-type counterparts (25, 32). AZT metabolites have been reported to inhibit HIV-1 integrase and RT RNAse H activities (33). Thus, it is reasonable to surmise that synergy between AZT and nevirapine in tissue culture assays may be related to aspects of AZT metabolite activity distinct from inhibition of HIV RT RNA-dependent DNA polymerization.

The sites and pattern of chain termination caused by nucleoside analogs were not affected by the presence of nevirapine (Fig. 2A). NNRTIs have been shown to block RT-mediated catalytic reactions without interfering with either nucleotide binding or nucleoside-induced conformational change (6, 27). Hence, NNRTIs may affect RT function without interfering with interactions between RT and template/primer.

Kinetic analysis revealed that combinations of nevirapine and delavirdine had an antagonistic effect with regard to inhibition of HIV RT. It is believed that NNRTIs bind to the same hydrophobic pocket within HIV RT (5, 27, 34). Our results, showing that nevirapine and delavirdine may antagonize each other’s binding, suggest that different chemical groups within the NNRTI binding pocket may mediate specific interactions with each drug, reflecting the fact that NNRTIs possess a multitude of distinct chemical structures. This is supported by the fact that HIV strains that are resistant to nevirapine may display cross-resistance to some NNRTIs but not to others (35), in spite of the fact that the mutations that encode resistance to NNRTIs are located around a common hydrophobic pocket (35).

Acknowledgment—We thank Michael Parniak for helpful discussions.

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J. Biol. Chem. 1995, 270:31046-31051.
doi: 10.1074/jbc.270.52.31046

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