Selective Action of 2',3'-Didehydro-2',3'-dideoxythymidine Triphosphate on Human Immunodeficiency Virus Reverse Transcriptase and Human DNA Polymerases*

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This study used DNA primer extension and sequencing gel analyses to evaluate the molecular action of 2',3'-didehydro-2',3'-dideoxythymidine triphosphate (D4TTP), in comparison with 3'-azido-2',3'-dideoxythymidine triphosphate (AZTTP), on DNA strand elongation by human immunodeficiency virus reverse transcriptases (HIV-RT) and human DNA polymerases α (pol α) and ε (pol ε) purified from T-lymphoblastoid CEM cells. D4TTP was preferentially incorporated into the T sites of the elongating DNA strand by HIV-RT and terminated DNA synthesis at the incorporation sites. The DNA chain termination activity of D4TTP was equipotent to that of AZTTP. In contrast, D4TTP was a poor substrate for pol α and pol ε. The analogue was incorporated into DNA by the human enzymes about 10,000- to 20,000-fold less efficiently than by HIV-RT, whereas the incorporation of AZTTP by pol α and pol ε was not detectable by the DNA primer extension assay. Pol ε, an enzyme with 3′ → 5′-exonuclease activity, was unable to remove the incorporated 2',3'-didehydro-2',3'-dideoxythymidine monophosphate (D4TMP) from the 3'-end of the DNA strand, whereas 3'-azido-2',3'-dideoxythymidine monophosphate was excised from DNA by pol ε at about 20% of the rate for normal deoxynucleotide excision. The preferential incorporation of D4TTP by HIV-RT appears to be a molecular basis for the selective anti-HIV activity of D4T, whereas the inability of pol ε to remove D4TMP from DNA may be related to the cytotoxicity of this compound.

The human immunodeficiency virus reverse transcriptase (HIV-RT) is the enzyme responsible for catalyzing the conversion of viral RNA to double-stranded DNA, which is then integrated into the host cellular DNA. Because HIV-RT plays an essential role in the viral life cycle, this enzyme is an important target for anti-retroviral chemotherapy. Most anti-HIV nucleoside analogues inhibit HIV replication by specifically targeting the viral reverse transcriptase. This action requires the metabolic conversion of the analogues to their active triphosphates. 3'-Azido-2',3'-dideoxythymidine (AZT), an analogue of thymidine, has been shown to be active in clinical treatment of patients with established HIV infection (2-4). It has been demonstrated that selective termination of DNA synthesis by HIV-RT by AZTTP is the major mechanism of the drug's action (5-12).

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Materials—D4TTP and AZTTP were chemically synthesized and purified according to the procedures described previously (13, 24). The M13 dideoxy sequencing kit was purchased from Bethesda Research Laboratories. The M13 mp18(+) strand DNA, the 17-base M13 sequencing primer (5′-dGTAAACGACGCTATG-3′), and ultrapure dATP, dCTP, dGTP, and dTTP were obtained from Pharmacia LKB Biotechnology Inc. HIV-RT was generously provided by Dr. R. T. C. Ting at Biotech Research Laboratories, Inc. The enzyme was purified from H9 cell cultures infected with HIV-1 by immuno-
Selective Action of D4TTP on HIV-RT

The DNA primer extension assay was used to evaluate the action of D4TTP on HIV reverse transcriptase and human pol α and pol ε. In Fig. 1, lane 1 contains a reaction with HIV-RT, dATP, dCTP, and dGTP without dTTP (T- reaction). The intense band at site 7 (1 nucleotide before the first T site) demonstrated that the majority of the 17-base primers were extended to and terminated at the site 1 nucleotide before the first T site, because there was no dTTP present in the reaction. The band at the first T site (site 8) indicated that some noncomplementary dNTP molecules were misincorporated into the T site by HIV-RT, confirming its error-prone properties (30–32). A small portion of the primer, containing misincorporated nucleotide, was further extended, resulting in the production of longer oligomers, as indicated by the two light bands at the sites 1 nucleotide before the third and fourth T sites.

When increasing concentrations of D4TTP were added to the T- reaction, the HIV-RT was able to efficiently use the analogue as the substrate for incorporation into the first T site (site 8) (Fig. 1, lanes 2–5). This incorporation led to a decrease in the number of primers at the pre-T site (site 7) and an accumulation of primers at the first T site. The incorporated D4TTP molecules terminated the primer extension; no DNA bands were detected beyond the first T site.

In the reactions that contained 30 μM each of dATP, dCTP, dGTP, and D4TTP, and various concentrations of dTTP (Fig. 1, lanes 6–9), dTTP competed with D4TTP for incorporation into the T sites and supported the synthesis of longer DNA strands. However, 30 μM dTTP was unable to reverse the chain termination activity of 30 μM D4TTP, no high molecular weight DNA strands were synthesized, and almost all primers were terminated within the first 15 T sites (lane 9).

In the experiments with 0.1 unit of purified human pol α and pol ε, higher concentrations of D4TTP (10–30 μM) were required to force the enzymes to incorporate trace amounts of D4TTP into the T sites (Fig. 2, lanes 4, 5, 14, and 15). The amount of HIV-RT, pol α, and pol ε used in this experiment produced approximately the same amount of high molecular weight DNA products in the absence of inhibitor, using the primed M13 mp18 DNA as the template. Quantitation of the radioactivity in this and another sequencing gel during a 100-min counting period showed that at 10 μM D4TTP, 830 ± 65 and 1172 ± 209 counts were incorporated into the first T site in the reactions with pol α and pol ε, respectively. In contrast, 4822 ± 455 counts were detected at the same site by HIV-RT when the D4TTP concentration was only 1 μM (Fig. 1, lane 1).
Selective Action of D4TTP on HIV-RT

The action of D4TTP or HIV-RT and pol α and pol ε was compared with that of AZTTP under identical conditions. As illustrated in Fig. 4, the incorporation of D4TTP and AZTTP into DNA by HIV-RT was similar under conditions measuring initial reaction rates over 30 min (lanes 2 and 3). Quantitation of two separate sequencing gels revealed that HIV-RT incorporated 22,912 ± 1497 counts and 24,876 ± 1498 counts into the first T site in reactions containing 30 μM D4TTP and AZTTP, respectively (lanes 2 and 3). A separate experiment demonstrated that the two analogues were incorporated by HIV-RT at similar, linear rates at 5-, 10-, 20-, 30-, 40-, and 60-min time points. Furthermore, the DNA chain termination patterns of D4TTP and AZTTP were identical (lanes 4 and 5). In the T' reaction (30 μM each of dATP, dCTP, and dGTP without dTTP) plus 30 μM D4TTP, 7775 ± 233 counts were incorporated into site 8 by pol α (lane 8), whereas 4373 ± 90 counts were detected in the reaction with 30 μM AZTTP (lane 9), which was not different from the background radioactivity in the T' reaction (4301 ± 22 counts, lane 7). Clearly, a small but statistically significant amount of D4TTP was incorporated by pol α (p < 0.01), whereas no significant amount of
AZTTP was incorporated ($p > 0.3$). Similarly phenomena were observed in reactions with pol $\epsilon$. A significant amount of D4TTP (5284 ± 111 counts, $p < 0.01$) was incorporated into the first T site by pol $\epsilon$ (lane 14), whereas the radioactivity (2823 ± 54 counts) in the reaction with AZTTP (lane 15) was not significantly different from that of the background (2644 ± 218, $p > 0.3$) (lane 13). Little effect was exhibited by either D4TTP or AZTTP on DNA primer elongation by pol $\alpha$ (lanes 10–12) or pol $\epsilon$ (lanes 16–18) when dTTP was present in the reaction mixtures.

To evaluate the ability of DNA pol $\epsilon$ to remove the incorporated D4TTP and AZTTP from the 3'-end of the DNA with its 3' → 5'-exonuclease activity, the 25-base oligomers with either D4TTP or AZTTP at the 3'-ends were constructed and annealed to the M13 mp18 (+) DNA. These DNA complexes were used as the substrates for excision by pol $\epsilon$. The normal 17-base primer was processed through the same procedures and used as the control. In Fig. 5, the upper panel demonstrates that during a 50-min incubation, pol $\epsilon$ removed a substantial amount of normal nucleotides from the 17-base primer, producing shorter oligomers (lanes 1–5). In contrast, the enzyme was unable to excise D4TTP from the 3'-end of the 25-base primer (lanes 8–12). When the primer with AZTMP at the 3'-end was used as the substrate, pol $\epsilon$ removed small amounts of the analogue from DNA at a slow rate (lanes 15–19). Quantitation of the radioactivity in the bands showed that AZTMP was removed at about 20% of the rate for normal nucleotide excision (Fig. 5, lower panel).

It was expected that if the DNA chain-terminating molecule (D4TMP or AZTMP) was removed from the 3'-end of the DNA primer by pol $\epsilon$, HIV-RT would be able to extend the primer to a high molecular weight DNA strand in the presence of four dNTPs. As illustrated in lane 6 of Fig. 5, HIV-RT extended the normal 17-base primer to high molecular weight DNA in the presence of dNTPs. When both HIV-RT and pol $\epsilon$ were included in the reaction containing the 17-base primer and four dNTPs, products of polymerization, as well as excision, were revealed (lane 7). The extension products appeared to be generated mainly by HIV-RT, because the natural pause band at about the 100-nucleotide site catalyzed by pol $\epsilon$ (Fig. 4, lane 18) was absent in this reaction (Fig. 5, lane 7). In the presence of pol $\epsilon$ and four dNTPs, however, HIV-RT was unable to extend the 25-base primer with 3'-D4TMP (lanes 13 and 14), indicating that the chain-terminating D4TMP residue still remained at the 3'-end of the primer after incubation with pol $\epsilon$. In contrast, pol $\epsilon$ was able to excise a small
amount of AZTMP from the 3'-end of the 25-base primer, which allowed the primer to be extended to high molecular weight DNA (lanes 20 and 21). Presumably, the excision products (24-base or smaller bands) were not detectable in those two reactions because the presence of four dNTPs enabled the extension of the short oligomers (<25 bases) to longer strands.

**DISCUSSION**

This study demonstrated that D4TTP was preferentially incorporated into DNA by HIV reverse transcriptase. In contrast, human DNA polymerases were unable to efficiently use D4TTP as the substrate for incorporation. Quantitative comparison showed that HIV-RT was about 10,000- to 20,000-fold more efficient than pol α and pol ε in incorporating D4TTP into DNA (Table I). This selective incorporation resulted in termination of DNA synthesis by the viral enzyme. Incubation of CEM cells with 2 μM D4T (23) or MT-4 cells with 1 μM D4T (19) for 24 h resulted in the accumulation of 1.4 and 0.22 μM cellular D4TTP, respectively. These concentrations are close to the K<sub>m</sub> value for D4TTP incorporation by HIV-RT (0.35 μM) and far below the K<sub>m</sub> values for human pol α (139.9 μM) and pol ε (156.6 μM). Thus, it may be important to monitor the cellular pharmacology of D4T. A D4T administration schedule that maintains cellular D4TTP at levels that terminate viral DNA synthesis but does not affect cellular DNA replication would optimize the therapeutic response.

The selective action of D4TTP on HIV-RT is similar to that of AZTTP (12). The comparison of their molecular action on DNA primer extension by HIV-RT revealed that D4TTP and AZTTP were incorporated into the growing DNA strand at the same efficiency and terminated DNA synthesis in the same manner. This is in agreement with the observation that D4TTP and AZTTP are equipotent in inhibiting HIV-RT (19). Both AZT and D4T are analogues of thymidine, and neither has a hydroxyl linked to the 3'-carbon of the sugar moiety. Their DNA chain termination activity is predicted because the 3'-hydroxyl is required for DNA strand elongation. The molecular mechanism of the selective action of these compounds on HIV-RT, however, is based on their preferential substrate efficiency for HIV-RT relative to human DNA polymerases. It appears that a thymidine analogue with a modification at the 3'-position is likely to be a preferred substrate for HIV-RT. Thus, molecular evaluation of the interaction between nucleotides bearing modifications at the 3'-position and HIV-RT and human DNA polymerases may be useful in the design of new anti-HIV drugs.

Although D4TTP is not a good substrate for human DNA polymerases, its incorporation into DNA by pol α and pol ε was detected in the DNA primer extension assay (Fig. 2). Thus, it is possible that this compound may be incorporated into cellular DNA in vivo, terminate chromosomal DNA replication, and ultimately lead to cytotoxicity. In contrast, no incorporation of AZTTP into DNA by human pol α and pol ε was detectable by DNA primer extension assay in this and other studies (12). The cytotoxic effects of D4T and AZT have been reported both in cell culture and in mice (13, 14, 21, 33). Because AZTTP is not a substrate for replicative human DNA polymerases, its inhibitory activity on DNA polymerase γ, a mitochondrial enzyme, may be in part responsible for its cytotoxicity (34). Furthermore, induction of alteration of cellular dNTP pools (35) and inhibition of DNA repair by AZT (36, 37) may also lead to cellular toxicity.

pol ε is a highly processive enzyme with 3' → 5' exonuclease activity (38, 39). This enzyme is the mammalian homologue of pol II in yeast (40) and is thought to be involved in both DNA replication (40) and repair (41, 42). The present study demonstrated that pol ε was able to effectively remove nucleotides from a normal 17-base DNA primer, but was unable to excise D4TTP from the 3'-end of an oligomer. AZTMP, however, was removed from the 3'-end of the DNA by pol ε at about 20% of the rate for normal deoxynucleotide excision. Thus, it is likely that incorporation of D4TTP or AZTMP into the DNA primer by HIV-RT results in a sterical change in the template-primer interaction that renders D4TTP impervious to and AZTMP less susceptible to excision by pol ε. The inability of pol ε to remove D4TTP from DNA may explain why the cytotoxic effect of D4T in H9 cells could not be reversed by addition of exogenous thymidine (33). Once the analogue is incorporated, it may remain in cellular DNA permanently. Whether DNA polymerase δ, another mammalian enzyme with 3' → 5' exonuclease activity, can remove D4TTP from DNA is not known. Detailed investigations of the excision of these analogues by pol β and pol ε in vitro and in whole cells will further our understanding of the drugs' actions.

**REFERENCES**

1. Wong-Staal, F. (1988) in HIV and Other Highly Pathogenic Viruses (Smith, R. A., ed) pp. 33-41, Academic Press, San Diego
2. Fischl, M. A., Richman, D. D., Grieco, M. H., Gottlieb, M. S., Volberding, P. A., Laskin, O. L., Leedom, J. M., Groopman, J. E., Mildvan, D., Schooley, R. T., Jackson, G. G., Durack, D. T., King, D., and the AZT Collaborative Working Group (1987) *N. Engl. J. Med.* 317, 185-191
3. Richman, D. D. (1988) *Infect. Dis. Clin. North Am.* 2, 397-407
4. Richman, D. D., Andrews, J., and the AZT Collaborative Working Group (1988) *Am. J. Med.* 85 (Suppl. 2A), 208-212
5. Furman, P. A., Fyle, J. A., St. Clair, M. H., Weinhold, K., Rideout, J. L., Freeman, G. A., Nusinoff-Lehrman, S., Bolognesi, D. P., Broder, S., Mituya, H., and Barry, D. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 8333-8337
6. Cheng, Y.-C., Dutschman, G. E., Bastow, K. F., Sarnagadharan, M. G., and Ting, R. Y. C. (1987) *J. Biol. Chem.* 262, 2187-2190
7. Vrang, L., Bazin, H., Remaud, G., Chattopadhyayya, J., and Oberg, B. (1987) *Antiviral Res.* 7, 139-149
8. St. Clair, M. H., Richards, C. A., Spector, T., Weinhold, K. J., Miller, W. H., Langlois, A. J., and Furman, P. A. (1987) *Antimicrob. Agents Chemother.* 31, 1972-1977
9. Bazin, H., Chattopadhyayya, J., Datema, R., Ericson, A.-C., Gilljam, G., Johansson, N. G., Hansen, J., Koshida, R., Moelling, K., Oberg, B., Remaud, G., Stening, G., Vrang, L., Wahren, B., and Wu, J. C. (1989) *Biochem. Pharmacol.* 38, 109-119
10. Sommadsos, J.-P., Carlisle, R., and Zhou, Z. (1989) *Mol. Pharmacol.* 36, 9-14
11. Furman, P. A., and Barry, D. W. (1988) *Am. J. Med.* 85 (Suppl. 2A), 176-181
12. Huang, P., Farquhar, D., and Plunkett, W. (1990) *J. Biol. Chem.* 265, 11914-11918
13. Mansuri, M. M., Starrett, J. E., Jr., Ghazzouli, I., Hitchcock, M. J. M., Sterzycki, R. Z., Brankovan, V., Johansson, N. G., Hansen, J., Koshida, R., Moelling, K., Oberg, B., Remaud, G., Stening, G., Vrang, L., Wahren, B., and Wu, J. C. (1990) *Biochem. Pharmacol.* 39, 2821-2829
14. Mansuri, M. M., Hitchcock, M. J. M., Buroker, R. A., Breman, C. L., Ghazzouli, I., Desiderio, J. V., Starrett, J. E., Sterzycki, R. Z., and Martin, J. C. (1990) *Antimicrob. Agents Chemother.* 34, 637-641
15. Lin, T.-S., Shinazi, R. F., and Prusoff, W. H. (1987) *Biochem. Pharmacol.* 36, 2713-2718
16. Hamamoto, Y., Nakashima, H., Matsui, T., Matsuda, A., Ueda, T., and Yamamoto, N. (1987) *Antimicrob. Agents Chemother.* 31, 907-910
17. Belmez, M., Pauwela, R., Herdewijn, P., De Clercq, E., Desmyter, J., and Van de Peer, M. (1987) *Biochem. Biophys. Res. Commun.* 142, 128-134
18. Balzarini, J., Kang, G.-J., Dalal, M., Herdewijn, P., De Clercq,
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E., Broder, S., and Johns, D. G. (1987) Mol. Pharmacol. 32, 162-167
19. Balzarini, J., Herdewijn, P., and De Clercq, E. (1989) J. Biol. Chem. 264, 6127-6133
20. Marongiu, M. E., August, E. M., and Prusoff, W. H. (1990) Biochem. Pharmacol. 39, 1523-1528
21. Gogu, S. R., Beckman, B. S., and Agrawal, K. C. (1989) Life Sci. 45, iii-vi
22. August, E. M., Birks, E. M., and Prusoff, W. H. (1991) Mol. Pharmacol. 39, 246-249
23. Ho, H.-T., and Hitchcock, M. J. M. (1989) Antimicrob. Agents Chemother. 33, 844-849
24. Harrington, J. A., Miller, W. H., and Spector, T. (1987) Biochem. Pharmacol. 36, 3757-3761
25. Veronese, F. M., Copeland, T. D., DeVico, A. L., Rahman, R., Oroszlan, S., Gallo, R. C., and Sarngadharan, M. G. (1986) Science 231, 1289-1291
26. Huang, P., Chubb, S., and Plunkett, W. (1990) J. Biol. Chem. 265, 16617-16625
27. Boosalis, M. S., Petruska, J., and Goodman, M. F. (1987) J. Biol. Chem. 262, 14698-14696
28. Parker, W. B., White, E. L., Shaddix, S. C., Ross, L. J., Buckheit, R. W., Jr., Germany, J. M., Secrist, J. A., III, Vince, R. V., and Shannon, W. M. (1991) J. Biol. Chem. 266, 1754-1762
29. Chou, J., and Chou, T. (1985) Dose-Effect Analysis with Microcomputers, pp. 5-18, Elsevier Science Publishers, Amsterdam
30. Preston, B. D., Poiesz, B. J., and Loeb, L. A. (1988) Science 242, 1168-1171
31. Takeuchi, Y., Nagumo, T., and Hoshino, H. (1988) J. Virol. 62, 3900-3902
32. Bebenek, K., Abbotts, J., Roberts, J. D., Wilson, S. H., and Kunkel, T. A. (1989) J. Biol. Chem. 264, 16948-16956
33. August, E. M., Marongiu, M. E., Lin, T.-S., and Prusoff, W. H. (1988) Biochem. Pharmacol. 37, 4419-4422
34. Chen, C.-H., Vazquez-Padua, M., and Cheng, Y.-C. (1991) Mol. Pharmacol. 39, 625-628
35. Frick, L. W., Nelson, D. J., St. Clair, M. H., Furman, P. A., and Krenitsky, T. A. (1988) Biochem. Biophys. Res. Commun. 154, 124-129
36. Munch-Petersen, B. (1988) Biochem. Biophys. Res. Commun. 157, 1309-1375
37. Scanlon, K. J., Kashani-Sabet, M., and Sowers, L. C. (1989) Cancer Commun. 1, 269-275
38. Bambara, R. A., and Jessee, C. B. (1991) Biochem. Biophys. Acta 1088, 11-24
39. Focher, F., Verri, A., Maga, G., Spadari, S., and Hubsher, U. (1990) FEBS Lett. 259, 349-352
40. Morrison, A., Araki, H., Clark, A. B., Hamatake, R. K., and Sugino, A. (1990) Cell 62, 1143-1151
41. Nishida, C., Reinhard, P., and Linn, S. (1988) J. Biol. Chem. 263, 501-510
42. Syvaoja, J., and Linn, S. (1989) J. Biol. Chem. 264, 2489-2497