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Activities of (−)-Carbovir and 3′-Azido-3′-Deoxythymidine against Human Immunodeficiency Virus In Vitro

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(−)-Carbovir, the minus optical isomer of carbocyclic-2′,3′-didehydro-2′,3′-dideoxyguanosine, has been shown to be the biologically active form for the inhibition of human immunodeficiency virus type 1 replication. The concentration of (−)-carbovir required to reduce reverse transcriptase activity by 50% compared with the control was 0.8 μM in H9 cells infected with the HTLV-IIIb strain; the 50% inhibitory concentration for cytotoxicity was >2 mM in these cells. The effect of the timing of drug addition, pre- and postinfection, and the effect of increasing amounts of virus on the antiviral activities of (−)-carbovir and 3′-azido-3′-deoxythymidine were determined.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of the acquired immunodeficiency syndrome (3, 4). Acquired immunodeficiency syndrome is characterized by the physical and functional depletion of the immune system of the body through the cytopathic infection of $CD4^+$ cells. The effective removal of these cells, primarily helper T cells and monocytes, from the host defense system results in an immune deficiency status, rendering the body susceptible to opportunistic infections, including *Pneumocystis carinii* infection, and to a rare neoplasm, Kaposi's sarcoma (5, 6, 23).

HIV-1 is a member of the *Lentivirinae* subgroup of the retrovirus family (8, 17). The retrovirus-specific reverse transcriptase has been a focus for the design of selective therapeutic agents, most notably derivatives of the naturally occurring nucleosides.

3′-Azido-3′-deoxythymidine (AZT) (7, 12, 21), currently the only drug marketed for the management of the HIV infection, and 2′,3′-dideoxycytidine (11) are very potent inhibitors of HIV replication. However, clinical trials with these drugs have revealed undesirable side effects (9, 13, 15, 16, 22), distinctly different for each drug, that may limit the clinical use of these agents. Consequently, other nucleoside analogs (e.g., 2′,3′-dideoxyadenosine and 2′,3′-dideoxyinosine [1] and 2′,3′-didehydro-2′,3′-dideoxythymidine [10; I. Ghazzouli, M. Hitchcock, V. Brankovan, J. Desiderio, J.-P. Sommadossi, M. August, T. S. Lin, W. Prusoff, M. Mansuri, and J. Martin, Second Int. Conf. Antiviral. Res., 1988]) with similar anti-HIV activity in vitro but less cytotoxicity to certain cell types are under development as promising new drugs for treatment of the acquired immunodeficiency syndrome.

Vince et al. (19) and White et al. (20) reported the antiviral effects of the carbocyclic analog of 2′,3′-didehydro-2′,3′-dideoxyguanosine, a compound known as carbovir (NSC 614846 or GI95146X). These initial data were described for the racemic mixture. We have found the minus enantiomer, (−)-carbovir, to be the biologically active isomer from the racemic mixture. We have characterized the antiviral activities associated with this isomer and compared them with those of AZT. The cells used for this study were the HIV-1-susceptible human T-cell line H9 (a gift from T. Matthews, Duke University Medical Center, Durham, N.C.). Cells were grown in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, penicillin [100 IU/ml], streptomycin [100 μg/ml], and glutamine [3 mg/ml]; GIBCO Laboratories, Grand Island, N.Y.) and screened routinely for *Mycoplasma* contamination. The HTLV-IIIb isolate of HIV-1 used for the infection of the H9 cells was obtained from the American Type Culture Collection, Rockville, Md. (ATCC CRL 8543). Viral stocks were quantitated for infectivity by using serial dilution analysis and assaying for the presence of reverse transcriptase activity in the culture supernatants. This established the dilution factor required to produce a detectable infection as the value for one tissue culture infectious dose for 50% of the isolates ($TCID_{50}$).

(1R-*cis*)-2-amino-1,9-dihydro-9[4-(hydroxymethyl)-2-cyclopenten-1-yl]-6H-purin-6-one [(−)-carbovir] (>98%) was synthesized at Glaxo; (+)-carbovir (>97%) was a gift from Robert Vince, University of Minnesota, and 3′-azido-3′-deoxythymidine (99%) was from Sigma Chemical Co., St. Louis, Mo. These antimicrobial agents were evaluated for antiviral activity by quantitating the reverse transcriptase activity in the culture supernatants. These assays were performed in a Costar flat-bottom 96-well plate coated with poly-L-lysine (2.5 μg per well; Sigma). H9 cells (2×10^6 to 5×10^6) were infected with HTLV-IIIb stock virus (0.01 $TCID_{50}$ per cell) for 1 h at 37°C. After incubation the cells were washed three times, and 5×10^4 cells were aliquoted into each well of the poly-L-lysine-conditioned, 96-well plate. Drug solutions were added to the well 18 h after the initial plating of the cells. Cells were incubated for 7 days and subsequently analyzed for reverse transcriptase activity in the culture supernatants as described by Poiesz et al. (14) and Spira et al. (18). Briefly, 100 μl of the culture supernatant was removed and treated with 11 μl of lysis buffer (0.5 M Tris hydrochloride [pH 8.0], 1.0 M NaCl, 5% Triton X-100, 10 mM EDTA), and 6 μl of the lysed sample was placed in the reverse transcriptase assay mixture (14, 18). The results of the reverse transcriptase activity are reported as the average of three replicates of the 6-μl samples.

The antiviral activities of (−)-carbovir were examined and compared with those of AZT in H9 cells infected with HTLV-IIIb (Fig. 1). Increasing concentrations of drug, ranging from 0.05 to 50 μM, were incubated with the infected cells for a period of 7 days and analyzed for the presence of reverse transcriptase activity in the culture supernatants.

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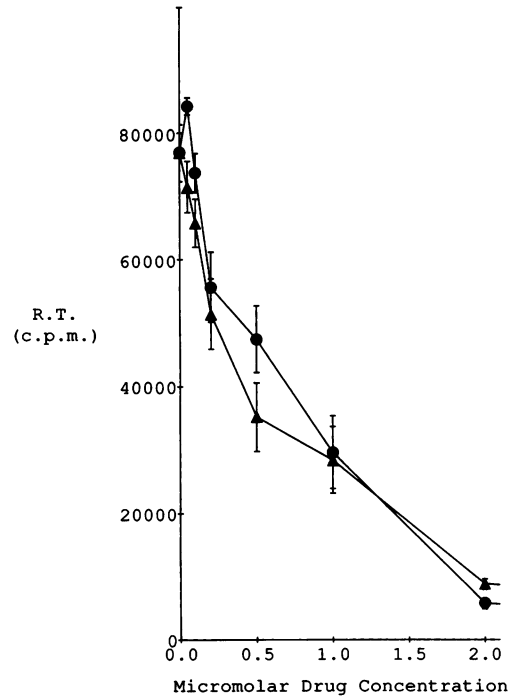

FIG. 1. Antiviral activities of (−)-carbovir (●) and AZT (▲) evaluated as a function of drug concentration in HTLV-IIIb-infected H9 cells for 7 days. Cell-free supernatants were analyzed for the presence of reverse transcriptase activity (R.T.). The error bars indicate the standard errors of the mean values for the replicates assayed.

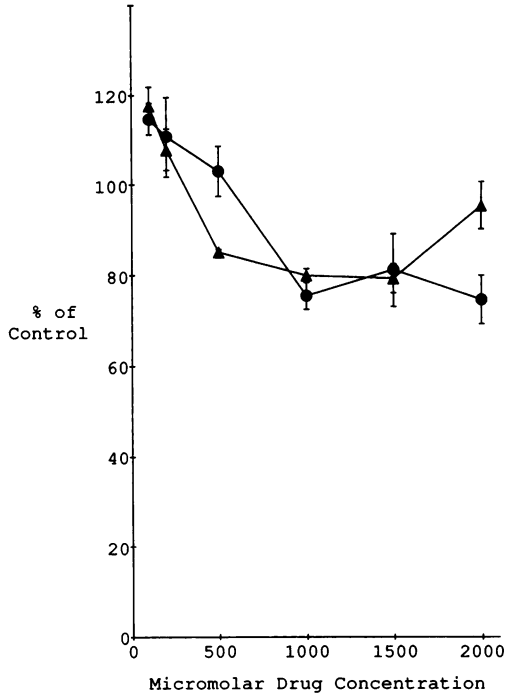

FIG. 2. Cytotoxicity of (−)-carbovir (●) and AZT (▲) to uninfected H9 cells (50,000 cells initially plated) was evaluated for 7 days by measuring cell viability with the MTT-Formazan dye conversion assay (2). The results are presented as percentages of the untreated control samples of H9 cells. The error bars indicate the standard errors of the mean values for the replicates assayed.

The profiles of the effects of the two drugs were similar for the inhibition of the replication of the virus. The concentration of drug required to reduce the reverse transcriptase activity by 50% relative to the untreated control for (−)-carbovir was 0.8 μM, compared with >60 μM for (+)-carbovir (data not shown) and 0.5 μM for AZT.

The cytotoxic effects of increasing concentrations of (−)-carbovir and AZT were evaluated in uninfected H9 cells by using a dye conversion cell viability assay (1). Figure 2 shows a cell viability profile of H9 cells incubated with concentrations of (−)-carbovir and AZT up to 2 mM for 7 days. Significant decreases (>50%) in cell viability were not observed over this concentration range; however, inhibitions of 25% were observed for both compounds at >1 mM.

The influence of the timing of drug addition to H9 cells, with respect to the time of infection with HIV-1, was investigated to assess the effects of early versus delayed treatment. H9 cells were incubated with 1 μM (−)-carbovir or 0.5 μM AZT at times ranging from 24 h before the cells were infected with HTLV-IIIb to 5 days postinfection. The cells were incubated continuously with the drug after the indicated initial exposure time point. (−)-Carbovir at 1 μM or AZT at 0.5 μM (approximately equivalent to the concentrations required to reduce the reverse transcriptase activity by 50% relative to the controls) were capable of inhibiting HIV-1 replication by 50% or greater when the drug was added up to 48 h postinfection (Fig. 3). The effectiveness of either drug was significantly greater when it was added to the cultures at times closer to the time of infection with HIV-1.

The effective antiviral activities of (−)-carbovir and AZT were tested as a function of the quantity of HIV-1 infectious units used for infection. There was a strong correlation between virus input and the effective antiviral activity of both (−)-carbovir and AZT (Fig. 4). When the inoculum was low (32 to 160 $TCID_{50}$ per 50,000 cells), inhibition was nearly complete for both agents in the range of drug levels tested. At the highest ratio of viral inoculum/multiplicity of infection tested (640 $TCID_{50}$), higher concentrations of both drugs were necessary to achieve the 50% inhibitory dose.

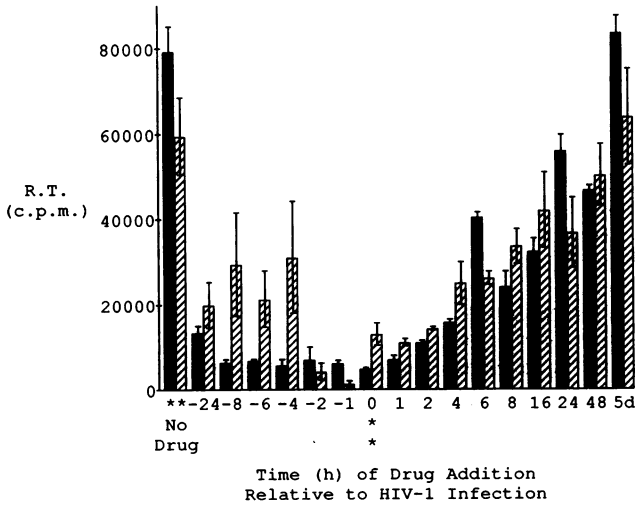

FIG. 3. Effects of the time of drug addition to H9 cells relative to the time of infection with HTLV-IIIb on the antiviral activity of 1 μM (−)-carbovir (■) or 0.5 μM AZT (▨). H9 cells were infected with HIV-1 at time zero with 0.01 $TCID_{50}$ per cell. The untreated control values were 79,198 cpm for (−)-carbovir and 67,647 cpm for AZT. R.T., Reverse transcriptase activity.

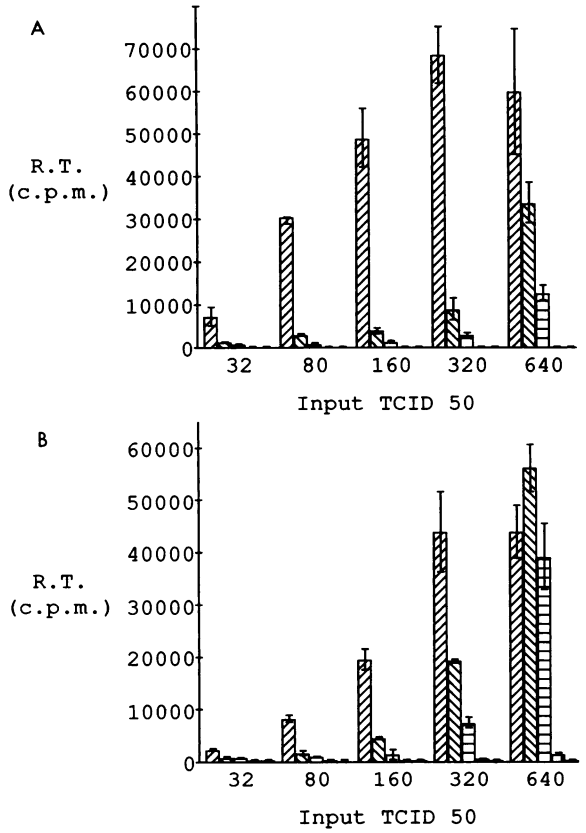

FIG. 4. Effects of increasing $TCID_{50}$ of HIV-1 on the antiviral activity of (−)-carbovir and AZT. H9 cells (50,000 cells) were infected with increasing amounts of HIV-1. The concentrations of (−)-carbovir (A) and AZT (B) at each viral inoculum were as follows: ▨, no drug; ▧, 0.5 μM; ▤, 1.0 μM; ▩, 10 μM and ■, 25 μM. The error bars represent the standard errors of the means for the replicates assayed. R.T., Reverse transcriptase activity.

This study has demonstrated that submicromolar concentrations of (−)-carbovir, the enantiomerically pure and biologically active form of carbovir (NSC 614846), effectively inhibit the replication of HIV-1 in H9 cells. The concentration required to elicit a cytotoxic effect of (−)-carbovir on this cell type is considerably greater (>1,000×). These results are similar to those obtained with AZT in parallel tests. The dose-response curves for (−)-carbovir and AZT were generated in cells with an active infection (Fig. 1). This condition reflects the ability of the drugs to block the replication of the virus after the viral metabolism is fully operational and may more closely represent the in vivo situation.

The effect of the viral inoculum or multiplicities of infection on the antiviral activity of (−)-carbovir and AZT was also examined in this study. Both drugs were effective inhibitors of viral replication at all levels of input virus tested, although there was a clear diminution of the antiviral effect with increasing multiplicities of HIV. The inhibitory response was furthermore affected by treatment delays, most notably when drug was added ≥48 h postinfection. However, the decline in the antiviral effect may relate to an active production of HIV during the drug-free incubation. This proliferation of virus during the drug-free phase may result in an apparent increase in the virus load and may contribute to the decline in the overall antiviral effect of the drugs.

We also performed initial testing of (−)-carbovir in other cell types (Molt-4, CEM-SS, THP-1, C3, MT-4, MT-2, U-937, CEM, C8166, JM, and primary human lymphocytes and monocytes) infected with a number of different isolates, including HTLV-IIIb, RF, GB8, lymphadenopathy-associated virus, and BaL and different clinical isolates of HIV-1 (unpublished data). The results from these additional experiments also demonstrate a potent antiviral effect with relatively low cytotoxicity. The inherently low cytotoxic effects of (−)-carbovir may be explained, at least in part, in terms of its apparent low affinity for a variety of human DNA polymerases and DNA primase activities, as was demonstrated with racemic carbovir (20). Further investigation is necessary to evaluate the use of (−)-carbovir for the treatment of the HIV infection.

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