

## Inhibitory Effect of 2',3'-Didehydro-2',3'-Dideoxynucleosides on Infectivity, Cytopathic Effects, and Replication of Human Immunodeficiency Virus

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It is generally accepted that human immunodeficiency virus (HIV) is the etiologic agent of the acquired immunodeficiency syndrome and related diseases. In this report, we demonstrate the antiviral effect of nucleoside analogs 2',3'-didehydro-2',3'-dideoxythymidine (DHT) and 2',3'-didehydro-2',3'-dideoxycytidine (DHC) by using human T-cell lymphotropic virus type I-carrying MT-4 cells, which are extremely susceptible to HIV infection. These agents efficiently inhibited the cytopathic effects and expression of HIV-specific antigens in MT-4 cells after infection of the virus. Both DHT and DHC also strongly blocked viral replication as determined by our quantitative bioassay system using a plaque-forming assay. These antiviral effects were obtained at concentrations at which the drugs produced little or no toxicity and were comparable to those with 3'-azido-3'-deoxythymidine and 2',3'-dideoxynucleosides. These findings warrant further investigation of the use of DHT and DHC for the treatment of the acquired immunodeficiency syndrome and related diseases.

Human immunodeficiency virus (HIV) replication may be essential for the pathogenesis of the acquired immunodeficiency syndrome (AIDS) and related diseases. Some kinds of antiviral agents, including ribavirin (8), suramin (10), antimoniungstate (16), and phosphonoformate (17), are considered possible therapeutics for AIDS. However, no therapy sufficient to cure the clinical manifestations of AIDS or restore the underlying immune deficiency has been found. Recently, it has been reported that nucleoside analogs, including 3'-azido-3'-deoxythymidine (AZT) and several 2',3'-dideoxynucleosides, had an inhibitory effect on the replication of human T-cell lymphotropic virus type III (HTLV-III), one of the strains of HIV (9, 11, 14). AZT was further examined clinically in 19 patients with AIDS-related complex for 6 weeks. The results of this study showed the safety and feasibility of administering AZT to patients with AIDS or AIDS-related complex and indicated that clinical conditions of some patients treated with AZT improved over 6 weeks. However, many issues still remained to be solved concerning the clinical use of this drug, especially for long-term administration (21). Therefore, it seems important to explore other effective drugs. Previously, we reported that HIV efficiently infected and propagated in a HTLV-I-carrying cell line, MT-4 (5, 12). This infection system provided a very effective way to screen possible anti-HIV drugs among a large number of candidates. Using MT-4 cells, we initially screened more than 50 nucleoside analogs for inhibitory effects on the HIV-induced cytopathic effect (CPE) as measured by [<sup>3</sup>H]thymidine uptake. Among them, two agents, 2',3'-didehydro-2',3'-dideoxythymidine (DHT) and 2',3'-didehydro-2',3'-dideoxycytidine (DHC), were picked for further examination. In this report, we demonstrate the inhibitory effect of these agents on HIV by using assays such as a trypan blue dye exclusion method for HIV-induced CPE, immunofluorescence (IF) for expression of HIV-specific antigens, and a plaque assay for quantitation of

biologically active virus. In addition, we studied in vitro antiviral and cytostatic effects of DHT and DHC compared with those of AZT, 2',3'-dideoxythymidine, and 2',3'-dideoxycytidine.

### MATERIALS AND METHODS

**Cells.** HTLV-I-carrying MT-4 cells were used in this study. The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU of penicillin per ml, and 100 µg of streptomycin per ml (complete medium) at 37°C in a CO<sub>2</sub> incubator and subcultured twice a week.

**Reagents.** DHT (Fig. 1a) was prepared from thymidine as described by Horwitz et al. (6). The melting point (164 to 165°C) and other physical properties of DHT were consistent with those previously reported (6). DHC (Fig. 1b) was prepared from 2'-deoxycytidine by a previously reported procedure (7). The melting point (167 to 169°C) and other physical properties of DHC were consistent with those previously reported (7). The purity of these compounds was determined by high-performance liquid chromatography using an Inertsil ODS column (5 m; 4.6 × 250 mm; Gasukuro Kogyo Inc., Tokyo, Japan) with 10% (vol/vol) aqueous methanol as eluent at a flow rate of 1.0 ml/min. The retention time and purity of these compounds were as follows: DHT, 23.3 min, >99%; and DHC, 8.8 min, >99%. AZT was prepared essentially as described by Glinski et al. (3). The purity of the AZT was more than 99.5%. 2',3'-Dideoxythymidine and 2',3'-dideoxycytidine were obtained from Pharmacia Fine Chemicals, Piscataway, N.J.

**Virus and virus infection.** The HTLV-III<sub>B</sub> isolate of HIV was obtained from the culture supernatant of its producer Molt-4/HTLV-III<sub>B</sub> cells as described previously (5). The titer of the viral preparation was 5 × 10<sup>4</sup> PFU/ml.

Infection of MT-4 cells with HIV was made at a multiplicity of infection of 0.001. For example, pelleted cells (5 × 10<sup>7</sup>) were mixed with 1 ml of virus suspension (5 × 10<sup>4</sup> PFU/ml) and incubated for 1 h at 37°C for adsorption of the virus.

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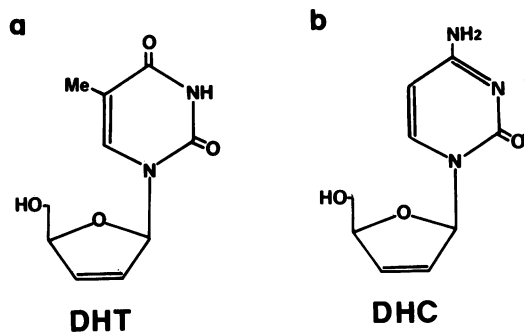


FIG. 1. Structures of DHT and DHC.

Then, infected cells were washed and suspended in complete medium with or without the reagents to make a concentration of  $3 \times 10^5$  cells per ml.

**Assay for HIV-induced CPE.** HIV-induced CPE was analyzed by measuring the decrease in the number of viable cells. The viable cells were counted by the trypan blue dye exclusion method.

**Indirect IF method.** Cell smears were dried and fixed with cold methanol and then stained by the indirect IF procedure as described previously (5). More than 500 cells were counted under a fluorescence microscope, and the percentage of IF-positive cells was calculated.

**Plaque-forming assay.** To determine the inhibitory effect of the reagents on HIV-induced plaque formation in MT-4 cells, a plaque-forming assay was performed as described previously (4, 13). Briefly, to fasten MT-4 cells onto culture vessels, 35-mm polystyrene tissue culture dishes were coated with poly-L-lysine ( $M_r$ , 120,000; Sigma Chemical Co., St. Louis, Mo.). MT-4 cells ( $1.5 \text{ ml}$ ;  $1.5 \times 10^5$  cells per ml) were dropped onto each poly-L-lysine-coated dish and incubated for 1 h at room temperature. The dishes were gently washed with phosphate-buffered saline (pH 7.4) to remove unbound cells, and  $100 \mu\text{l}$  of diluted virus preparation (2,000 PFU/ml) was slowly added over the cells. The cells were incubated for 1 h at room temperature for virus adsorption. After incubation, various concentrations of chemicals in 1.5 ml of the agarose overlay medium consisting of RPMI 1640 medium with 10% fetal bovine serum, antibiotics, and 0.6% agarose (Sea Plaque agarose; FMC Corp., Marine Colloids Div., Rockland, Maine) were poured onto each dish. The dishes were incubated in a  $\text{CO}_2$  incubator at  $37^\circ\text{C}$  for 3 days, and 1.5 ml of agarose overlay medium containing neutral red was added. The dishes were incubated at  $37^\circ\text{C}$  for 3 more days, and visible plaques were counted. All experiments were done in triplicate.

## RESULTS

**Effect of DHT and DHC on growth of MT-4 cells.** We measured the growth inhibitory effects of the reagents on MT-4 cells which were not exposed to the virus. The viable cell growth of MT-4 cells treated with 0, 1, 2, 10, 25, and 50  $\mu\text{g}$  of DHT and DHC per ml is shown in Fig. 2a and b, respectively. Viable cells were counted on day 3 (open bar) and day 6 (lined bar) in culture by the trypan blue dye exclusion method. Significant growth inhibition of MT-4 cells was observed at DHT concentrations of 25  $\mu\text{g}/\text{ml}$  (119  $\mu\text{M}$ ) and 50  $\mu\text{g}/\text{ml}$  (Fig. 2a) and DHC concentrations of 10  $\mu\text{g}/\text{ml}$  (51  $\mu\text{M}$ ), 25  $\mu\text{g}/\text{ml}$ , and 50  $\mu\text{g}/\text{ml}$  (Fig. 2b).

**Inhibitory effect on HIV-induced CPE.** To investigate the inhibitory effects of the reagents on HIV-induced CPE, DHT

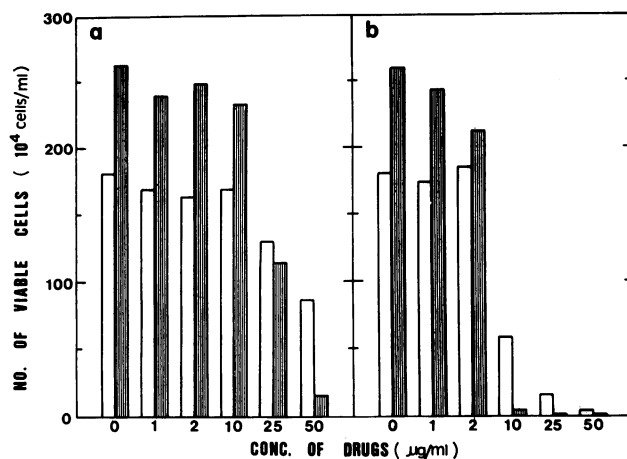


FIG. 2. Effects of DHT (a) and DHC (b) on growth of MT-4 cells. MT-4 cells were cultured in the presence or absence of various concentrations of DHT and DHC. The viable cells were counted on days 3 (open bar) and 6 (lined bar) by a trypan blue dye exclusion method. Representative results of three experiments are shown.

and DHC were used in these experiments at concentrations of less than 10  $\mu\text{g}/\text{ml}$  and less than 2  $\mu\text{g}/\text{ml}$ , respectively. By HIV infection, the viable MT-4 cells were decreased gradually as reported (4). In the present experimental conditions (multiplicity of infection, 0.001), the number of viable cells dropped to  $4 \times 10^4/\text{ml}$  after 6 days of incubation. By contrast, in the media containing more than 0.1  $\mu\text{g}$  of DHT per ml (Fig. 3a) and more than 0.2  $\mu\text{g}$  of DHC per ml (Fig. 3b), the MT-4 cells remained proliferative. These results suggest that both DHT and DHC have a strong protective effect against HIV-induced CPE at the concentrations of 0.1 to 10 and 0.2 to 2  $\mu\text{g}/\text{ml}$ , respectively.

**Inhibitory effect on expression of HIV antigens.** When MT-4 cells were infected by HIV, 47% of the cells became positive for virus antigens 3 days after infection and 100% became

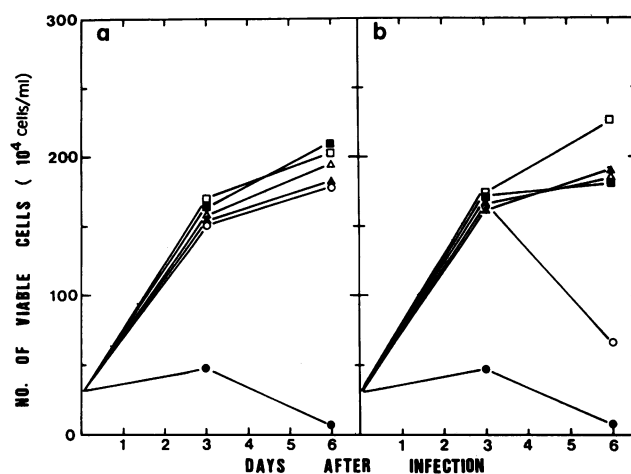


FIG. 3. Effect of nucleoside analogs on growth of MT-4 cells after HIV infection. HIV-infected MT-4 cells were cultured in the presence or absence of various concentrations of agents. (a) DHT:  $\square$ , 10  $\mu\text{g}/\text{ml}$ ;  $\blacksquare$ , 1  $\mu\text{g}/\text{ml}$ ;  $\triangle$ , 0.5  $\mu\text{g}/\text{ml}$ ;  $\blacktriangle$ , 0.2  $\mu\text{g}/\text{ml}$ ;  $\circ$ , 0.1  $\mu\text{g}/\text{ml}$ ;  $\bullet$ , 0. (b) DHC:  $\square$ , 2  $\mu\text{g}/\text{ml}$ ;  $\blacksquare$ , 1  $\mu\text{g}/\text{ml}$ ;  $\triangle$ , 0.5  $\mu\text{g}/\text{ml}$ ;  $\blacktriangle$ , 0.2  $\mu\text{g}/\text{ml}$ ;  $\circ$ , 0.1  $\mu\text{g}/\text{ml}$ ;  $\bullet$ , 0. Representative results of three experiments are shown.

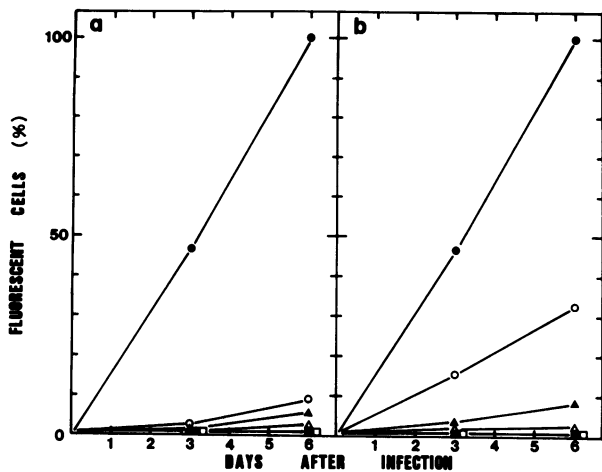


FIG. 4. Inhibitory effect of nucleoside analogs on expression of HIV-specific antigen. HIV-infected MT-4 cells were cultured in the presence or absence of various concentrations of agents. The percentage of HIV-specific antigen expressed in cells was determined by the indirect IF method. (a) DHT: □, 10 µg/ml; ■, 1 µg/ml; △, 0.5 µg/ml; ▲, 0.2 µg/ml; ○, 0.1 µg/ml; ●, 0. (b) DHC: □, 2 µg/ml; ■, 1 µg/ml; △, 0.5 µg/ml; ▲, 0.2 µg/ml; ○, 0.1 µg/ml; ●, 0. Representative results of three experiments are shown.

positive 6 days after infection, as determined by an indirect IF technique (Fig. 4). A significant inhibition of HIV antigen synthesis was observed in a dose-dependent manner when HIV-infected MT-4 cells were cultured in the presence of DHT (Fig. 4a) and DHC (Fig. 4b). In particular, no virus-antigen-positive cell was detected at concentrations of 1 to 10 µg of DHT per ml and 1 and 2 µg of DHC per ml by 6 days after infection.

**Inhibition of HIV-induced plaque formation.** To evaluate the effect of the reagents on HIV-induced plaque formation in MT-4 cells, various concentrations of the chemicals were added to the agarose medium directly after infection. When 0.05 µg of DHT (Fig. 5a) and DHC (Fig. 5b) per ml was added to the agarose, only 21 and 91 plaques were observed, respectively, whereas 216 plaques were formed in control dishes. Moreover, more than 0.1 µg of both reagents per ml

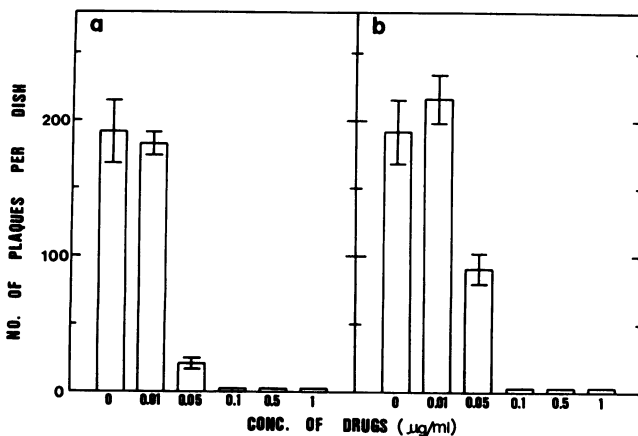


FIG. 5. Inhibitory effect of DHT (a) and DHC (b) on HIV-induced plaque formation in MT-4 cells. Experiments were done in triplicate. The numbers (mean ± standard deviation) of plaques formed at the various concentrations in the overlay medium are shown.

TABLE 1. Comparative potency of anti-HIV substances as revealed by IF in HIV-infected MT-4 cells

Compound	ID <sub>50</sub> <sup>a</sup> (µg/ml)
AZT	0.005
DHT	0.009
DDC	0.065
DHC	0.080
DDT	3.86

<sup>a</sup> On day 6 after infection, experiments were performed in duplicate by the IF method as described in Materials and Methods. Numbers represent the concentrations of anti-HIV substances to inhibit 50% of viral activity, which were derived by plotting the mean percentages of inhibition, which were calculated as follows: [1 - (percent IF-positive cells in the presence of inhibitor/percent IF-positive cells in the absence of inhibitor)] × 100. DDT, 2',3'-Dideoxythymidine; DDC, 2',3'-dideoxycytidine.

completely inhibited plaque formation. We also examined, by means of a plaque-forming assay, the number of infectious virus particles released from 4-day-old cultures of HIV-infected MT-4 cells in the presence of various concentrations of the reagents. Although  $25 \times 10^4$  plaques per ml were calculated in the control virus preparation obtained from HIV-infected MT-4 cells, the addition of DHT or DHC resulted in a decrease in the number of plaques in a dose-dependent fashion. Virus samples from the cultures treated with more than 2 µg of DHT or 1 µg of DHC per ml did not form plaque at all (data not shown).

**Antiviral effects of DHT and DHC in comparison with those of AZT, 2',3'-dideoxythymidine, and 2',3'-dideoxycytidine.** To assess the extent of activities of DHT and DHC, they were compared with AZT, 2',3'-dideoxythymidine, and 2',3'-dideoxycytidine in the same assay system. On day 6 after infection (multiplicity of infection, 0.001), significant inhibition of viral antigen expression was observed with AZT and DHT. Both reagents showed 50% inhibitory doses (ID<sub>50</sub>) for the induction of viral antigens of 0.005 and 0.009 µg/ml, respectively. DHC, 2',3'-dideoxycytidine, and 2',3'-dideoxythymidine were less potent, showing ID<sub>50</sub> of 0.080, 0.065, and 3.86 µg/ml, respectively (Table 1). The inhibitory effects of these compounds on HIV-induced CPE corresponded well to effects on viral antigen expression (data not shown). Under these experimental conditions, each nucleoside showed no cytostatic effect on growth of MT-4 cells not infected with HIV.

DISCUSSION

In the present study, we showed that DHT and DHC produced anti-HIV effects at concentrations from 0.1 to 10 and 0.2 to 2 µg/ml, respectively. These agents efficiently protected against HIV-induced CPE in MT-4 cells. The drugs also blocked the expression of virus-specific antigens in MT-4 cells after infection with virus. Moreover, a plaque-forming assay, which gives a better estimate of inhibitory effects on viral replication, showed that these nucleoside analogs also completely blocked viral replication at the concentration of 2 µg/ml in MT-4 cells. In addition, we performed a plaque assay by using overlay agarose including various concentrations of nucleoside analogs to evaluate the direct effect of agents on virus-induced plaque formation in MT-4 cells. When more than 0.1 µg of these agents per ml was added to the agarose overlay medium, no plaque was detectable, whereas about 200 plaques were formed in control dishes. It is important to note that no growth inhibition of uninfected MT-4 cells was evident within the effective concentrations described above.

Our results show that DHT was superior to DHC in inhibiting viral antigen expression, as shown in Table 1. Very recently, Balzarini et al. also reported that DHC inhibited the infectivity and replication of HIV in human lymphoid cell lines, including MT-4 cells (1). With respect to DHC, the results of the present studies roughly matched those reported by Balzarini et al. (1). The mechanism of action of these agents is unknown. Our experiments showed that there seems to be no direct effect on the virus particles by these nucleoside analogs (data not shown). It is known that the 5'-triphosphates of dideoxynucleosides, such as 2',3'-dideoxyadenosine, -dideoxyguanosine, -dideoxycytidine, and -dideoxythymidine, can rather easily inhibit cellular DNA polymerases  $\beta$  and  $\gamma$ , as well as viral reverse transcriptase, but no mammalian DNA polymerase  $\alpha$  (2, 15, 18-20). Both DHT and DHC are also 2',3'-dideoxynucleosides and have double bonds between the 2' and 3' positions. Thus, it is possible that they may also be converted to triphosphates to inhibit viral reverse transcriptase in cells. Moreover, the double bonds between the 2' and 3' positions possibly play an important role in antiviral effect. Further studies are required to determine the exact mechanism of action of DHT and DHC in their anti-HIV activity.

It is unknown whether these in vitro observations regarding the antiviral activity of DHT and DHC will correlate with clinical efficacy in humans. There are reports suggesting that some dideoxynucleosides, such as dideoxyadenosine, have rather negligible effects on the growth of cultured mammalian cells (9, 20). The present study suggests that DHT and DHC have a relatively low cytotoxicity. However, the relative potencies and cytotoxicities of these compounds may differ in other cell types. Further evaluation of these drugs is required before they can be investigated for use in the treatment of HIV infections in vivo.

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