Greatly Enhanced Inhibition of Human Immunodeficiency Virus Type 1 Replication in CEM and HT4-6C Cells by 3'-Deoxythymidine Diposphate Dimyristoylglycerol, a Lipid Prodrug of 3'-Deoxythymidine

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3'-Deoxythymidine (3dT) is a weakly active dideoxynucleoside in human immunodeficiency virus (HIV)-infected cells because of its slow phosphorylation by cellular thymidine kinase. 3dT diphosphate dimyristoylglycerol (3dTDP-DMG), a phospholipid prodrug, was synthesized and found in vitro to be 18- to 50-fold more effective than 3dT in CEM and HT4-6C cells. In CEM cells, the selectivity index of 3dTDP-DMG was 270 versus 48 for 3dTMP, an increase of 5.6-fold. In thymidine kinase-deficient mutant CEM cells infected with HIV, 3dT and zidovudine (AZT) were virtually inactive but 3dTDP-DMG retained substantial activity, suggesting that its greatly increased antiviral activity is due in part to bypass of thymidine kinase. 3dTDP-DMG was 14- to 37-fold more active than 3dT in AZT-sensitive and AZT-resistant clinical isolates of HIV; no cross-resistance with AZT was noted. The results suggest that lipid prodrugs may be utilized in some cases to confer unique metabolic advantages over the corresponding free nucleoside; in the case of 3dTDP-DMG, an 18- to 50-fold increase in antiretroviral activity was observed in LAV-infected cells. The strategy would seem to be especially useful for antiviral nucleosides which are poorly phosphorylated.

The human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS (3, 6, 10). Current therapies presently in clinical use for AIDS are the dideoxynucleoside inhibitors of HIV reverse transcriptase such as 3'-azido-3'-deoxythymidine (zidovudine [AZT]), 2',3'-dideoxynosine (ddl) and ddC (12). AZT and the other dideoxynucleosides inhibit HIV reverse transcriptase after conversion to the triphosphate (5).

Of the dideoxynucleosides originally tested in ATH8 cells by Mitsuya and Broder, the least active was 3'-deoxythymidine (3dT; dideoxythymidine), which required 100 to 200 μM concentrations to inhibit HIV replication by 50% (IC50) (11). In contrast, other dideoxynucleosides (ddA, ddC, ddG, and ddl) had IC50s of 0.1 to 10 μM (11). Similar results in ATH8 cells were reported by Heredewijn et al. (7) and Balzarini et al. (2). In MT4 cells infected with HIV, 3dT was relatively more effective, with an IC50 of 0.2 μM, but this activity was minimal compared with that of AZT, which had an IC50 of 0.006 μM (1). In our previous studies with HIV-infected U937 and CEM cells (8), 3dT was a poor inhibitor of HIV replication, with IC50s of >100 and >32 μM, respectively.

As part of our interest in finding compounds which can be targeted to HIV-infected macrophages, we found that phosphatidyl-3dT, a lipid prodrug of 3dT, exhibited increased activity in HIV-infected CEM and U937 cells, compared with that of free 3dT (8). To further investigate the increased activity exhibited by lipid prodrugs, we synthesized 3dT diphosphate dimyristoylglycerol (3dTDP-DMG). This phospholipid conjugate has an sn-1,2-DMG-3-pyrophosphate group attached to the 5'-hydroxyl of 3dT. This report describes greatly increased biological activity of this compound versus that of the free nucleoside in both HIV-infected human CEM T lymphoblasts and thymidine kinase (TK)-deficient CEM T-lymphoblast mutants. Possible explanations for the enhanced biological activity of 3dTDP-DMG are proposed.

MATERIALS AND METHODS

Synthesis and purification of 3dTDP-DMG and phosphatidyl-3dT. 3dT (dideoxythymidine) was obtained from Sigma Chemical, St. Louis, Mo. 1,2-Dimyristoylphosphatidyl-3dT was prepared as described previously (8). 3dTMP was prepared by reacting 3dT with phosphorus oxychloride essentially as described previously by Yoshikawa et al. (17, 18) and purified by using a Q-Sepharose column eluted with a gradient of 0.1 M NH4HCO3 (pH 7.2) as described by van Wijk et al. (14, 15).

3dTDP-DMG was synthesized by a new method which gives higher yields and facilitated purifications; details of the procedure will be reported elsewhere (16). Briefly, 1,2-dimyristoylphosphatidic acid (Avanti, Birmingham, Ala.) was converted to the morpholidate by reacting it with morpholine in the presence of dicyclohexylcarbodiimide in t-butanol. After removal of the solvents in vacuo, the resulting dimyristoylphosphatic acid morpholidate (5 to 10% molar excess) was allowed to react with 3dTMP (free acid) in dry pyridine for 20 h at room temperature. After removal of the solvents in vacuo, the residue was dissolved in a minimum volume of chloroform-methanol (1/1) and applied to a semipreparative silica column (0.19 by 30 cm) (Porasil; Waters, Milford, Mass.), which was eluted with hexane-isopropanol-25% ammonia-water (43/57/3/7 [by volume]) at flow rates of 12 to 16 ml/min. The fractions containing 3dTDP-DMG were pooled, and a white powder was obtained.

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after removal of the solvent. The purified compound gave a single spot with an Rf of 0.29 when applied to silica gel G thin-layer plates developed with chloroform-methanol-25% ammonia-water (70/38/8/2 [by volume]). The compound was stable when stored dry for at least 3 months at −20°C. Dimyristoylphosphatidyl-3dT was synthesized and purified as described previously (8).

**Phosphatidyl-3dT and 3TDP-DMG formulation.** Liposomes containing dioleylphosphatidylcholine, cholesterol, phosphatidylglycerol and 3TDP-DMG (or dimyristoylphosphatidyl-3dT) (molar ratio, 50/30/10/10) were prepared by sonication in 0.3 ml of sterile 10 mM sodium acetate buffer (pH 5.0) containing isotonic dextrose as previously described (8). The final liposome suspension had a nominal 3dT-liponucleotide concentration of 5 mM. The preparations were diluted with sterile RPMI buffer and added to the tissue culture wells at the indicated concentrations. In one experiment, 3TDP-DMG was dissolved in dimethyl sulfoxide (DMSO) and added to tissue culture wells at concentrations of from 0.1 to 100 μM and the final concentration of DMSO was adjusted to 0.2% in each well.

**In vitro assessment of antiviral activity.** (i) CEM cells and HIV. The human lymphoblastoid line CEM-CCRF (American Type Culture Collection, Rockville, Md.) was grown in RPMI 1640 medium containing 100 U of penicillin G per ml, 100 μg of streptomycin per ml, 2 mM glutamine, and 10% fetal bovine serum (HyClone Laboratories, Logan, Utah). The cells were infected with HIV-1BRU (L. Montagnier, Paris, France) at a multiplicity of infection of one 50% tissue culture infective dose per cell for 60 min at 37°C in medium containing 1% polybrene. CEM cells were infected in a suspension at 6 × 10⁶ cells per ml, washed three times by centrifugation and resuspension, and then distributed in 96-well plates at 6 × 10⁴ cells per well before addition of medium containing the liposomal antiretroviral liponucleotide drugs.

(ii) HIV p24 assay. Antiviral activity was measured after 3 days of incubation as the reduction of p24 antigen in the cell-free supernatant from the cultured cells which had been exposed to the indicated concentrations of drugs. The p24 antigen was measured in duplicate by enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories, Chicago, Ill.) according to the manufacturer’s instructions. The data are expressed as percentages of the control incubated without drug. Under the conditions of the assay, CEM cells produced viral p24 antigen logarithmically to a peak at 3 days which was roughly 1,000 times greater than residual inoculum levels, with 2.4 to 4 μg/ml or greater in culture supernatants.

(iii) HT4-6C cells and plaque reduction assay. CD4-expressing HeLa cells, HT4-6C cells (4), were obtained from Bruce Chesebro, Hamilton, Mont. The effect of antiviral compounds on HIV replication was measured by a plaque reduction assay. Briefly, monolayers of HT4-6C cells were infected with 100 to 300 PFU of virus per well in 24-well microdilution plates. Various concentrations of drug were added to the culture medium, Dulbecco’s Modified Eagle Medium containing 5% fetal bovine serum and antibiotics, as noted above. After 3 days at 37°C, the monolayers were fixed with 10% formaldehyde solution in phosphate-buffered saline and stained with 0.25% crystal violet to visualize virus plaques (9). Antiviral activity was assessed as the percentage of control plaques measured in drug-treated samples.

(iv) Selection of TK-deficient CEM T lymphoblasts. A variant of the human CEM T-lymphoblastoid cell line deficient in hypoxanthine phosphoribosyltransferase was mutagenized by exposure to ethyl methanesulfonate and then grown in 100 mM 5-fluoro-2'-deoxyuridine. Surviving cells were cloned by limiting dilution and assayed for TK activity radiochemically as described previously (13).

**RESULTS**

**Antiviral activity in HT4-6C cells.** Free 3dT reduced plaque formation by 50% at 90 μM in HT4-6C cells infected with HIV-1 (Fig. 1). However, 3TDP-DMG caused 50% inhibition at 1.8 μM, representing a 50-fold increase in antiviral activity. A lipid control without 3dT liponucleotide had no effect on plaque formation at a lipid concentration equivalent to that of the 100 μM liponucleotide samples (a total lipid concentration of 1 mM). The liponucleotide was equally active in liposomal formulation or as the free 3TDP-DMG in 0.2% DMSO. At 0.1 μM, liposomal and DMSO formulations of 3TDP-DMG reduced HT4-6C plaque formation relative to untreated HIV-infected controls by 51% ± 3% and 53% ± 6%, respectively (n = 3).

**Antiviral activity in wild-type CEM T lymphoblasts.** The antiviral activities of 3dT and 3TDP-DMG were assessed in CEM cells infected with HIV-1BRU (Fig. 2). The lipid produg was formulated in liposomes and incubated with HIV-infected CEM cells for 3 days, and the levels of p24 in the supernatant were determined by ELISA. 3dT was a very weak inhibitor of p24 production (IC₅₀, 29 μM). 3TDP-DMG was 18 times more active in HIV-1-infected CEM cells, with 50% inhibition of p24 production noted at 1.6 μM. As noted earlier, phosphatidyl-3dT was three times more effective than free 3dT, with an IC₅₀ of 14 μM (8). A phospholipid liposome control without 3dT liponucleotide and matched for the amount of total lipid per well had no effect on p24 production over a wide range of concentrations, in agreement with our earlier observations (8).

**Determination of toxicity and selectivity index.** To assess...
the effect of the 3dT liponucleotides on cell viability, we incubated uninfected CEM and HT4-6C cells with 3dTDP-DMG or phosphatidyl-3dT at concentrations ranging from 0.1 to 1,000 μM and determined viable cell number by trypan blue exclusion. Phosphatidyl-3dT reduced viable cell number by 50% (TD50) at 820 μM, while 3dTDP-DMG had a TD50 of 430 μM (Table 1). In both cases, this was due primarily to effects on cell number; cell viability was not affected by drug concentrations of up to 1 mM. Control lipids had no effect on the number of viable CEM cells at lipid concentrations matched to 1,000 μM liponucleotide. Free 3dT was less toxic to CEM cells than the liponucleotides (TD50, 1,380 μM), consistent with its poor initial phosphorylation and conversion to 3dTTP. The selectivity index for 3dT was 48 versus 60 and 270 μM for phosphatidyl-3dT and 3dTDP-DMG, respectively.

In HT4-6C cells treated with 3dTDP-DMG, no toxicity was apparent by visual observation and trypan blue staining of the cell monolayers at liponucleotide concentrations of up to 1,000 μM. The selectivity index of 3dTDP-DMG in this cell line is >556.

**Characterization of TK-deficient human CEM T lymphoblasts.** TK-deficient mutants of CEM T lymphoblasts were selected as noted in Materials and Methods. As determined by radiochemical assay, the fluorodeoxyuridine-resistant CEM mutant had less than 1% of wild-type TK activity in cell extracts. Similarly, viable cells incorporated less than 1% as much [5-3H]thymidine into DNA as did parental cells. These two cell types were used to compare the antiviral activities of 3dT and of the liponucleotides of 3dT.

**Activity in HIV-infected TK-deficient CEM mutants.** 3dT had little effect on p24 production by HIV-1-infected TK− CEM cells. However, 3dTDP-DMG reproducibly inhibited p24 production by HIV-infected TK− CEM cells, as shown in Fig. 3. The IC50 of 3dTDP-DMG was about sixfold higher (10.1 μM) in the TK− CEM cell line than in the wild-type CEM cells, suggesting that phosphorylation by TK was not required for its antiviral action. The data from TK− cells suggested that the greatly increased activity of 3dTDP-DMG may have been due, at least in part, to its ability to bypass TK because of its unique cellular metabolism. Phosphatidyl-3dT was inactive in TK− cells, suggesting that it did not give rise to cellular 3dTMP. (Fig. 3).

TK-deficient CEM T lymphoblasts infected with HIV were also resistant to AZT; no inhibition of p24 production was observed at AZT concentrations of up to 100 μM. However, AZT diphosphate DMG inhibited HIV p24 production by 50% in TK− CEM cells at concentrations of 5 to 10 μM (data not shown), providing further support for direct cellular metabolism of nucleoside diphosphate DMGs to nucleoside monophosphates.

**3dTDP-DMG activity in AZT-sensitive and AZT-resistant HIV clinical isolates.** 3dTDP-DMG and 3dT were compared in HT4-6C cells infected with AZT-sensitive and AZT-resistant

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**TABLE 1. Effect of 3dT liponucleotides on HIV replication and viable cell number in CEM cells in vitro**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
<th>TD50 (μM)</th>
<th>Selectivity index (TD50/IC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3dT</td>
<td>29</td>
<td>1,380</td>
<td>48</td>
</tr>
<tr>
<td>Phosphatidyl-3dT</td>
<td>14</td>
<td>820</td>
<td>60</td>
</tr>
<tr>
<td>3dTDP-DMG</td>
<td>1.6</td>
<td>430</td>
<td>270</td>
</tr>
</tbody>
</table>

* IC50, concentration of drug required to inhibit p24 production by 50% in 3 days; TD50, concentration of drug required to reduce viable cell number by 50% after a 3-day incubation. Untreated CEM cells increased from 3 x 10^4 to 12.1 x 10^4 ± 2.3 x 10^3/ml (n = 9) at day 3 of incubation (97% viable). The drug-induced reductions in viable cell numbers were due entirely to decreased cell numbers at the respective TD50. Cell viability was not decreased significantly below liponucleotide concentrations of 1 mM.

† Phosphatidyl-3dT, dimyristoylphosphatidyl-3dT.

‡ Datum taken from reference 8.
TABLE 2. Effect of 3dT and 3dTDP-DMG on AZT-sensitive and AZT-resistant clinical isolates of HIV in HFT4-6C cells, in vitro

<table>
<thead>
<tr>
<th>Isolate</th>
<th>AZT status</th>
<th>IC50 (µM)</th>
<th>3dT</th>
<th>3dTDP-DMG</th>
</tr>
</thead>
<tbody>
<tr>
<td>A012B</td>
<td>Sensitive</td>
<td>0.01</td>
<td>200</td>
<td>8</td>
</tr>
<tr>
<td>A012D</td>
<td>Resistant</td>
<td>2</td>
<td>290</td>
<td>20</td>
</tr>
<tr>
<td>A018A</td>
<td>Sensitive</td>
<td>0.01</td>
<td>220</td>
<td>6</td>
</tr>
<tr>
<td>A018C</td>
<td>Resistant</td>
<td>4</td>
<td>185</td>
<td>14</td>
</tr>
</tbody>
</table>

* The clinical isolates and the AZT data were first reported by Larder et al. (9). The data for 3dT and 3dTDP-DMG are the averages of two experiments.

3dTDP-DMG is 18 to 50 times more effective than 3dT, in vitro. The precise metabolic reason for the increased activity may be due both to increased cell uptake and to metabolic differences. The studies with TK-deficient CEM T-lymphoblast mutants showed that 3dTDP and AZT diphosphate diglycerides were effective even when cellular TK was essentially absent, suggesting that AZT diphosphate DMG and 3dTDP-DMG can be metabolized directly to AZT monophosphate or 3dTMP, bypassing TK. The exact metabolic explanation for the greatly increased antiviral activity of the liponucleotide diphosphate diglyceride in CEM and HT4-6C cells was not explored. However, the increased resistance to 3dT (Table 2), 3dTDP-DMG was somewhat less active in the clinical isolates than in the HIV-1_BRU laboratory strain of HIV (IC50 of 6 to 20 versus 1.6 µM, respectively), but the lipid produg of 3dT was 14 to 37 times more active than 3dT in the AZT-sensitive and AZT-resistant clinical isolates.

**DISCUSSION**

3dTDP-DMG is 18 to 50 times more effective than 3dT, in vitro. The precise metabolic reason for the increased activity may be due both to increased cell uptake and to metabolic differences. The studies with TK-deficient CEM T-lymphoblast mutants showed that 3dTDP and AZT diphosphate diglycerides were effective even when cellular TK was essentially absent, suggesting that AZT diphosphate DMG and 3dTDP-DMG can be metabolized directly to AZT monophosphate or 3dTMP, bypassing TK. The exact metabolic explanation for the greatly increased antiviral activity of the liponucleotide diphosphate diglyceride in CEM and HT4-6C cells was not explored. However, the increased resistance to 3dT (Table 2), 3dTDP-DMG was somewhat less active in the clinical isolates than in the HIV-1_BRU laboratory strain of HIV (IC50 of 6 to 20 versus 1.6 µM, respectively), but the lipd produg of 3dT was 14 to 37 times more active than 3dT in the AZT-sensitive and AZT-resistant clinical isolates.

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