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 (3Td) is a weakly active dideoxynucleoside in human immunodeficiency virus (HIV)-infected cells because of its slow phosphorylation by cellular thymidine kinase. 3Td diphasphate dimyristoylglycerol (3dTDP-DMG), a phospholipid prodrug, was synthesized and found in vitro to be 18- to 50-fold more effective than 3Td in CEM and HT4-6C cells. In CEM cells, the selectivity index of 3dTDP-DMG was 270 versus 48 for 3Td, an increase of 5.6-fold. In thymidine kinase-deficient mutant CEM cells infected with HIV, 3Td 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 3Td 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'-dideoxyinosine (ddI) 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 (3Td; dideoxthymidine), which required 100 to 200 μM concentrations to inhibit HIV replication by 50% (IC50) (11). In contrast, other dideoxynucleosides (ddA, ddC, ddG, and ddI) had IC50s of 0.1 to 10 μM (11). Similar results in ATH8 cells were reported by Herdewijn et al. (7) and Balzarini et al. (2). In MT4 cells infected with HIV, 3Td 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), 3Td 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-3Td, a lipid prodrug of 3Td, exhibited increased activity in HIV-infected CEM and U937 cells, compared with that of free 3Td (8). To further investigate the increased activity exhibited by lipid prodrugs, we synthesized 3Td diphasphate dimyristoylglycerol (3dTDP-DMG). This phospholipid conjugate has an sn-1,2-DMG-3-pyrophosphate group attached to the 5'-hydroxyl of 3Td. 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. 3Td (dideoxthymidine) was obtained from Sigma Chemical, St. Louis, Mo. 1,2-Dimyristoylphosphatidyl-3Td was prepared as reported previously (8). 3dTMP was prepared by reacting 3Td 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 morpholinate by reacting it with morpholine in the presence of dicyclohexylcarbodiimide in t-butanol. After removal of the solvents in vacuo, the resulting dimyristoylphosphatic acid morpholinate (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 (43/3/7 [by volume]) at flow rates of 12 to 16 ml/min. The fractions containing 3dTDP-DMG were pooled, and a white powder was obtained.
variant of cence control plaques (9). Antiviral activity was assessed with 10% without drug. Under the Medium containing plates. Various assay. reduction pounds on HIV replication was measured viral cago, assay munosorbent ing HeLa data are expressed as cell-free infective units. 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 x 10⁶ cells per ml, washed three times by centrifugation and resuspension, and then distributed in 96-well plates at 6 x 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 immunosorbert 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 0.2% 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 muta-

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, 3dTDP-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 3dTDP-DMG in 0.2% DMSO. At 0.1 µM, liposomal and DMSO formulations of 3dTDP-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 3dTDP-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). 3dTDP-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
FIG. 2. 3dT and 3dTDP-DMG inhibition of p24 antigen production by HIV-1-infected CEM T-lymphoblastoid cells. Values are means ± standard deviations. (n = 3). Symbols are as described in the legend to Fig. 1.

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 × 10⁶ to 12.1 ± 2.3 × 10⁶/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 TD₅₀s. Cell viability was not decreased significantly below liponucleotide concentrations of 1 mM. Phosphatidyl-3dT, dimyristoylphosphatidyl-3dT.

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 3DTP. 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.

FIG. 3. Effect of 3dT liponucleotides on HIV replication in TK-negative CEM T-lymphoblastoid cells. Values are means ± standard deviations (n = 3). Closed circles, 3dTDP-DMG; open triangles, dimyristoylphosphatidyl-3dT; open circles, 3dT.

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 [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 3TMP. (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 diphasphate 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 diphasphate 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 virus isolates first reported by Larder et al. (9). The AZT-resistant isolates were 200 to 400 times less sensitive to AZT in vitro (9). 3dT was weakly active in AZT-sensitive clinical isolates, and the resistant isolates did not exhibit any cross-resistance to 3dT (Table 2). 3dTDP-DMG was somewhat less active in the clinical isolates than in the HIV-1BRU laboratory strain of HIV (IC\textsubscript{50} of 6 to 20 versus 1.6 \textmu M, respectively), but the lipid prodrug 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-lymphoblastoid 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 is not clear, as a comparative study of cellular levels of the two liponucleotides and their metabolites, including the mono-, di-, and triphosphates of 3dT.

However, in other experiments with membrane fractions isolated from liver, we found that 3dTDP-DMG could substitute for CDP-DG as a substrate for mitochondrial phosphatidylglycerol biosynthesis, providing one possible metabolic explanation for direct formation of 3dTMP (14). Subsequent experiments showed that the amount of 3dTMP formed was much too large to be accounted for by phosphatidylglycerol synthesis alone. Further study with a \textsuperscript{3}H-labeled substrate showed direct hydrolysis of 3dTDP-DMG to phosphatidic acid and \textsuperscript{[3]}H\textsubscript{3}dTMP by a mitochondrial pyrophosphatase (15).

Although further study in host cells such as CEM, CEM TK\textsuperscript{-}, and HT4-6C and in human monocyte/macrophages is required, it seems likely that 3dTDP-DMG is 18 to 50 times more active than 3dT, at least in part because the liponucleotide can be metabolized directly to 3dTMP, bypassing slow phosphorylation of 3dT by TK. 3dTDP-DMG is also active in AZT-resistant strains of HIV and could be useful clinically in treating HIV infection.

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