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Antiviral Research 24 (1994) 59–67

Antiviral Research

# Antiviral activity of phosphatidyl-dideoxycytidine in hepatitis B-infected cells and enhanced hepatic uptake in mice

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(Received 17 November 1993; accepted 22 February 1994)

**Abstract**

Dideoxycytidine (ddC) inhibits the replication of hepatitis B virus (HBV) but its clinical use is limited by peripheral neuropathy. We synthesized dioleoylphosphatidyl-ddC (DOP-ddC), a phospholipid prodrug of ddC which forms lipid bilayers and is readily incorporated into liposomes. The 90% effective dose ($ED_{90}$) of DOP-ddC was 18 $\mu$M vs. 7 $\mu$M for ddC. However, in HBV-infected human hepatoma cells (2.2.15 cells), DOP-ddC was less toxic in vitro. When liposomal DOP-[5,6-$^3$H]ddC was administered intraperitoneally to mice, drug levels in liver were 40 times greater than [5,6-$^3$H]ddC when expressed as area under curve. Liposomal DOP-ddC also provided higher levels of drug in lymph nodes and spleen, important accessory sites of HBV replication. Plasma levels of drug remained above the $ED_{90}$ six times longer with DOP-ddC than with ddC. DOP-ddC levels in sciatic nerve, the major site of toxicity, were not significantly different from levels observed with free ddC. The phospholipid prodrug approach is a general one which may readily be applied to other antiviral nucleosides for HBV.

*Key words:* Hepatitis B; Antiviral agent; Phosphatidyl-dideoxycytidine; Prodrug; Phospholipid; Liposome

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*SSDI* 0166-3542(94)00022-Z

## 1. Introduction

Dideoxynucleosides inhibit the replication of HIV and other retroviruses (Chaisson et al., 1988; Fischl et al., 1987; Fischl et al., 1990; Mitsuya et al., 1985; Spector et al., 1989; Volberding et al., 1990; Yarchoan et al., 1986). Dideoxycytidine (ddC) is a potent inhibitor of HIV replication. It decreases serum p24 levels and improves CD4 cell counts in AIDS patients (Merigan et al., 1989; Mitsuya and Broder, 1986; Yarchoan et al., 1988). However, ddC therapy is associated with a dose-related sensory peripheral neuropathy which limits its utility (Merigan et al., 1989; Yarchoan et al., 1988).

DdC and other dideoxynucleosides also inhibit the replication of duck hepatitis B virus (DHBV) in vitro (Lee et al., 1989) and in vivo (Kassianides et al., 1989) and HBV in the human hepatoblastoma cell line, 2.2.15 (Aoki-Seki et al., 1991; Korba and Milman, 1991; Lampertico et al., 1991). Recently a number of analogs of dideoxycytidine and dideoxythymidine have been shown to have antiviral activity in 2.2.15 cells, in vitro (Doong et al., 1991; Korba and Gerin, 1992; Matthes et al., 1992; Furman et al., 1992). Dideoxycytidine and dideoxyguanosine, appear to have a substantial degree of efficacy and selectivity in 2.2.15 cells, in vitro (Korba and Gerin, 1992).

To treat hepatitis B more effectively, it would be desirable to direct a larger fraction of administered antiviral nucleosides to the liver because some of these agents are toxic to nonhepatic tissues and may not achieve adequate levels in liver tissue. To increase nucleoside uptake by the liver, we synthesized dioleoylphosphatidyl-dideoxycytidine (DOP-ddC), the dioleoylphosphatidic acid conjugate of ddC. Like other phosphatidyl-nucleosides (Hostetler et al., 1990), DOP-ddC forms lipid bilayers in the presence of other lipids such as phosphatidylcholine, phosphatidylglycerol and cholesterol. The antiviral activity of liposomal dispersions of DOP-ddC was evaluated in 2.2.15 cells in vitro and tissue distribution in liver, spleen and lymph node was determined using DOP-(5,6-$^3$H]ddC.

## 2. Materials and Methods

*Synthesis of phosphatidyl-ddC.* Dioleoylphosphatidyl-ddC (1,2-dioleoyl-*sn*-glycero-3-phospho-5′-(2′,3)-dideoxycytidine, DOP-ddC) was synthesized by coupling 100 mg of *sn*-1,2 dioleoylphosphatidic acid (0.13 mmol; Avanti Biochemicals, Birmingham, AL) to 28 mg ddC (0.13 mmol; Sigma Chemicals, St. Louis, MO.) using triisopropylbenzenesulfonyl chloride (170 mg, 0.56 mmol; Aldrich, Milwaukee, WI) in dry pyridine as previously described for phosphatidyl-AZT (Hostetler et al., 1990). The compound was purified on a column containing 5 g of silica-gel (Kieselgel 60, E. Merck, Darmstadt), successively eluted with 10 to 20% methanol in chloroform as previously described (Hostetler et al., 1990) and the fractions containing the product were combined and concentrated to afford the desired product as a single UV and phosphorous positive spot ($R_f$ 0.42, Analtech Silica gel plates, $CHCl_3$/MeOH/$NH_3$/$H_2O$, 70:30:1:1). Other spectral data were consistent with the

structure, $^1$H (nmr, $CDCl_3$) $\delta$ ppm; 0.88 (t, 6H, $CH_3$), 1.26 (m, 44H, $CH_2$), 1.57 (brs, 4H, -CO-$CH_2$), 1.8 and 2.26 (brs, 4H, H′$_2$ and H′$_3$), 2.00 (m, 4H, $\beta$-CH′$_2$), 2.8 (m, 4H, $CH_2$-$CH_2$CH-), 4.1–4.4 (m, 7H, $CH_2$-*sn*-1,3, H′$_4$, H′$_5$), 5.34 (m, 6H, CH=CH, CH-*sn*-2, $H_4$), 6.00 (brs, 1H, H′$_1$), 6.5 (brs, 2H, $NH_2$), 7.04 (m, 1H, $H_5$). HPLC analysis showed the product to be >97% pure (Jones Diol Column, RT 11.7 min, 4.6 × 250 mm (5$\mu$), 1 ml/min, solvent A: 95/5 (THF/1 mM $NaH_2PO_4$), solvent B: 20/80 (THF/10 mM $NaH_2PO_4$), A→ 5 min, 100% B in 20 min.

*DOP-ddC liposomes for in vitro antiviral studies.* DOP-ddC was incorporated into sonicated vesicles containing dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidyl-glycerol (DOPG), cholesterol (Chol) at a molar ratio of DOPC/DOPG/Chol/Drug of 50:10:30:10 in sterile isotonic dextrose containing 10 mM sodium acetate (pH 5.0) as previously described (Kumar et al., 1992). DdC or DOP-ddC was added to the culture medium, at the concentrations indicated. Lipid controls matched for total lipid content (DOPC/DOPG/Chol, 60:10:30 mol %) were incubated similarly.

*2.2.15 cells and assessment of drug effects on HBV-specific DNA.* Free ddC, lipid controls and sonicated liposomes containing DOP-ddC were prepared as described above and were used to treat confluent cultures 2.2.15 cells (Sells et al., 1988) for 10 days. Details of the assay methodology were previously reported (Korba and Gerin, 1992).

*Tissue levels of [$^3$H]ddC and DOP-[$^3$H]ddC after intraperitoneal administration to mice.* 2′,3′-[5,6$^3$H]ddC was obtained from Moravek Biochemicals, Brea, CA. DOP-[5,6$^3$H]ddC was synthesized and purified as previously described for phosphatidyl-AZT (Hostetler et al., 1991) and liposomes consisting of DOPC/Chol/DOP-[5,6$^3$H]ddC (67:30:3 mol %) were prepared by 4 cycles of extrusion through 200 nm stacked polycarbonate filters (Mayer et al., 1986). DOP-[5,6$^3$H]ddC or [5,6$^3$H]ddC were injected intraperitoneally into mice at a dose of 15 $\mu$mol/kg (3 mg/kg) ddC or 15 $\mu$mol/kg of DOP-[5,6$^3$H]ddC). The mice were sacrificed at the times indicated and plasma, liver, spleen and lymph node were removed and processed for liquid scintillation counting. The results were expressed as nmol tritium-labeled ddC and ddC metabolites/gm tissue ± S.D. ($n$ = 3).

## 3. Results

*AntiHBV activity of DOP-ddC and ddC.* Dideoxycytidine and liposomal DOP-ddC were incubated with 2.2.15 cells at concentrations ranging from 3 to 100 $\mu$M (Table 1). Both 30–100 $\mu$M free ddC and $\mu$M DOP-ddC caused a reduction of HBV-specific DNA in the culture medium to undetectable levels by day 10. Persistent viral DNA was noted in the culture medium on day 10 at 3 $\mu$M ddC and 10 $\mu$M DOP-ddC. Cellular levels of HBV-specific DNA replicative intermediates and HBV virion DNA in the culture medium on day 10 were progressively reduced by increasing concentrations of free ddC and DOP-ddC; the doses which reduced HBV-specific DNA in the culture medium by 90% ($ED_{90}$) were 7 $\mu$M and 18 $\mu$M for free ddC and DOP-ddC, respectively. A lipid control without DOP-ddC had no effect on HBV

Table 1
HBV replication in 2.2.15 cell cultures: effect of ddC and dioleoylphosphatidyl-ddC

| Treatment | Intracellular HBV DNA [pg/μg cell DNA ± S.D.] | | | HBV DNA in culture medium [pg/ml ± S.D.] | | | |
|---|---|---|---|---|---|---|---|
| | Integrated | Monomers | Rep.Int. | day 0 | day 3 | day 6 | day 10 |
| Untreated cells | 1.1 ± 0.1 | 2.1 ± 0.2 | 73 ± 14 | 84 ± 22 | 75 ± 27 | 65 ± 26 | 79 ± 18 |
| 100 μM ddC | 1.2 ± 0.2 | 0.6 ± 0.4 | 0.1 ± 0.1 | 77 ± 17 | 27 ± 5 | 1 ± 1 | 0 |
| 30 μM ddC | 1.2 ± 0.1 | 0.8 ± 0.3 | 1 ± 0.4 | 70 ± 17 | 28 ± 6 | 4 ± 1 | 0 |
| 10 μM ddC | 1.0 ± 0.1 | 1.3 ± 0.5 | 6 ± 1 | 89 ± 14 | 64 ± 17 | 20 ± 6 | 2 ± 1 |
| 3 μM ddC | 1.2 ± 0.4 | 2.0 ± 0.2 | 19 ± 3 | 61 ± 3 | 51 ± 2 | 50 ± 8 | 27 ± 4 |
| 100 μM DOP-ddC | 1.1 ± 0.1 | 0.5 ± 0.1 | 0.2 ± 0.1 | 82 ± 7 | 20 ± 5 | 0 | 0 |
| 30 μM DOP-ddC | 1.1 ± 0.2 | 0.6 ± 0.3 | 1 ± 0.3 | 74 ± 16 | 51 ± 5 | 15 ± 2 | 0 |
| 10 μM DOP-ddC | 1.1 ± 0.1 | 1.8 ± 0.2 | 45 ± 6 | 80 ± 4 | 90 ± 30 | 61 ± 13 | 25 ± 8 |
| 100 μM Lipid control | 1.1 ± 0.2 | 2.2 ± 0.1 | 80 ± 11 | 58 ± 17 | 44 ± 11 | 60 ± 17 | 70 ± 8 |

Values presented are the mean and standard deviations (S.D.). When no S.D. value is given, all values in the indicated category were identical. Values for HBV DNA replicative intermediates (Rep. Int.) and culture medium (virion) DNA have been rounded to two significant figures. Abbreviations: ddC, 2',3'-dideoxycytidine; DOP-ddC, 1,2-dioleoyl-glycero-*sn*-3-phospho-5'-dideoxycytidine. Levels of integrated HBV DNA, which do not have a direct role in HBV replication, are expected to remain constant on a per CD1 basis (Korba and Gerin, 1992). These HBV DNA levels are used to control for lane to lane differences in total DNA levels.

DNA at a concentration of 100 μM (Table 1).

Toxicity was assessed by neutral red uptake (Table 2). Free ddC reduced neutral red uptake by 50% ($TD_{50}$) at 210 μM; DOP-ddC was less toxic, with a $TD_{50}$ of 1000 μM. The lipid control without DOP-ddC had no effect on neutral red uptake at a concentration equivalent to 1000 μM DOP-ddC (10 mM total lipid). In rapidly dividing CEM cells, the $TD_{50}$ for ddC and DOP-ddC was 2.6 and 31.6 μM, respectively (Hostetler et al., unpublished data).

*Tissue distribution of [$^3$H]ddC and DOP-[$^3$H]ddC in mice.* 15 μmol/kg tritium-labeled ddC and 15 μmol/kg liposomal DOP-[$^3$H]ddC was administered by intraperitoneal injection in mice and plasma and tissue levels of [$^3$H]ddC were determined at various times. Plasma, liver and sciatic nerve concentrations are shown in Fig. 1. Free dideoxycytidine levels were highest in plasma at 15 min (15.8 μM), and declined rapidly to less than 0.34 and 0.20 μM by 4 and 6 h, respectively. In contrast, the peak level of DOP-ddC in plasma was 49 μM at 1 h declining more gradually to 7.9 and 6.0 μM at 4 and 6 h. In liver peak drug levels were 15 nmol/gm for ddC at 15 min vs. 78 nmol/gm at 1 h with DOP-ddC. DOP-ddC levels in liver declined much more slowly over the first 12 h to 4.2 nmol/gm while ddC levels were less than 0.3 nmol/gm in liver after only 4 h. Sciatic nerve levels of ddC and DOP-ddC were low and declined rapidly to 0.12±0.14 and 0.33±0.07 at 6 h. At 24 h DOP-ddC levels were 0.01 nmol/gm in sciatic nerve. The differences between ddC and DOP-ddC levels in sciatic nerve were not statistically significant.

Table 2
Toxicity analysis of ddC and dioleoylphosphatidyl-DdC in 2.2.15 cells

| Compound | Neutral red dye uptake at indicated drug concentration (% of control) | | | |
|---|---|---|---|---|
| | 1000 μM | 300 μM | 100 μM | 30 μM |
| ddC | 2 ± 1 | 24 ± 4 | 98 ± 2 | 101 ± 2 |
| DOP-ddC | 48 ± 3 | 99 ± 2 | 100 ± 1 | 100 ± 2 |
| Lipid control | 97 ± 3 | 100 ± 1 | 101 ± 2 | 101 ± 4 |

Toxicity analyses were performed in 96-well flat bottomed tissue culture plates (Korba and Gerin, 1992). Cells for the toxicity analyses were cultured and treated with test compounds with the same schedule as used for the antiviral evaluations. Each compound was tested at 4 concentrations, each in triplicate cultures. Uptake of neutral red dye was used to determined the relative level of toxicity. The absorbance of internalized dye at 510 nM ($A_{510}$) was used for the quantitative analysis. Values are presented as a percentage of the average $A_{510}$ values (± S.D.) in 9 separate cultures of untreated cells maintained on the same 96-well plate as the test compounds. The percentage of dye uptake in control cultures was 100 ± 4. Abbreviations: ddC, 2',3'-dideoxycytidine; DOP-ddC, 1,2-dioleoyl-*sn*-glycero-3-phospho-5'-dideoxycytidine.

## 4. Discussion

DOP-ddC exhibited substantial antiviral activity in 2.2.15 cells which chronically produce HBV. While the antiviral activity of DOP-ddC was lower than ddC in vitro (Table 1), its toxicity was substantially less of that of ddC (Table 2). The in vitro selectivity index ($TD_{50}/ED_{90}$) of ddC was 30 vs. 56 for DOP-ddC. The reasons for the improved selectivity of DOP-ddC are not presently clear and will require studies of 2.2.15 cell uptake and metabolism of ddC and DOP-ddC.

Liposomal DOP-[$^3$H]ddC administered intraperitoneally to mice resulted in 40-fold higher area under curve (AUC) values in liver than those observed with an equimolar dose of intraperitoneal [$^3$H]ddC. In spleen and lymph node, accessory sites of hepadnavirus replication (24), DOP-[$^3$H]ddC produced 86- and 2.9-fold higher AUC values than ddC. We calculated the percentage of the total administered dose of ddC or DOP-ddC in the liver at the time of peak levels (15 min for ddC and 1 h for DOP-ddC). At 15 min, 4.9% of the administered ddC was recovered in liver vs. 31% for DOP-ddC at 1 h. In sciatic nerve, the toxicity target of ddC, no significant increase in AUC was noted with DOP-[$^3$H]ddC and its metabolites. Finally, it is possible to roughly estimate the in vivo time course of antiHBV activity by calculating the elapsed time until the plasma levels of ddC and metabolites falls below the $ED_{90}$. Using the $ED_{90}$ data for ddC (7μM) and DOP-ddC (8μM) (Table 1) and the levels of ddC and DOP-ddC in plasma (Fig. 1) time above $ED_{90}$ is 30 min for ddC vs. about 3 h for DOP-ddC.

Our results suggest that parenteral administration of phospholipid prodrugs of antiviral nucleosides may increase the in vivo efficacy and selectivity of nucleoside therapy for hepatitis B infection by delivering a much larger fraction of the administered drug to the liver. Further studies are required to evaluate what proportion of the dose is delivered to hepatic parenchymal cells vs. endothelial and Kupfer cells in

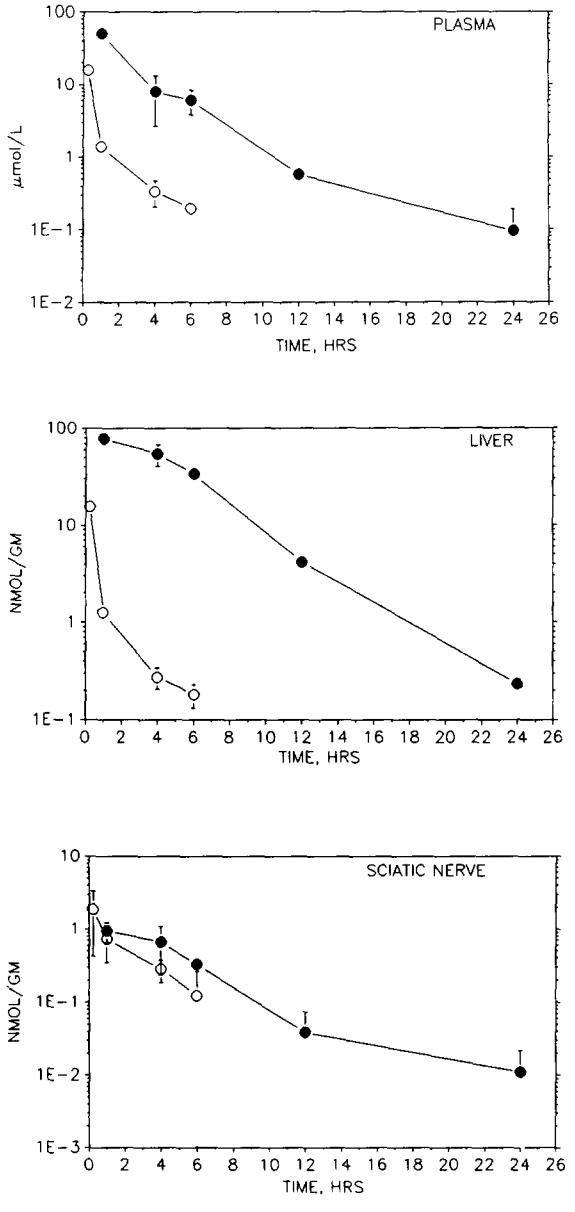


Fig. 1. Plasma, liver and sciatic nerve levels of [5,6-$^3$H]ddC and DOP-[5,6-$^3$H]ddC following intraperitonal administration in mice. Open circles, 15 μmol/kg free [5,6-$^3$H]ddC; closed circles, 15 μmol/kg DOP-[5,6-$^3$H]ddC. Results represent the mean ± S.D. of 3 separate determinations done in duplicate.

vivo. However, previous studies by Scherphof and coworkers with liposomes containing tritium-labeled inulin have shown that substantial hepatic parenchymal cell uptake occurs depending on liposome lipid composition and size (Spanjer et al.,

1986). The predicted benefits of phospholipid prodrug targeting to liver may be assessed directly in woodchuck hepatitis B (WHBV) infection in vivo by comparing the efficacy and toxicity of free and targeted nucleosides.

Targeting liposomal phospholipid prodrugs of antiHBV nucleosides to liver is a general approach which can be applied to any antiviral nucleoside. Many nucleosides active against HBV are known to be very toxic in vivo and antiviral glycerophospholipid prodrugs targeted to liver might be expected to reduce drug toxicity and improve efficacy if the liver itself is not the toxicity target. Recently, (−)-3′-thiadideoxycytidine (3TC) and (−)-5′-fluoro-3′-thiadideoxycytidine (FTC), analogs of ddC, have been shown to have excellent antiHBV activity in vitro in 2.2.15 cells (Doong et al., 1991; Furman et al., 1992); we are currently preparing phospholipid prodrug analogs of 3TC and dideoxyguanosine for further evaluation.

In conclusion, DOP-ddC was synthesized by conjugating dioleoylphosphatidic acid to the 5′-hydroxyl of dideoxycytidine and the phospholipid prodrug was shown to have antiviral activity in 2.2.15 cells, in vitro. Although DOP-ddC was somewhat less effective in vitro than free ddC, its toxicity in 2.2.15 cells was only 20% of that of free ddC and its in vitro selectivity index was 56 vs. 30 for ddC. Intraperitoneal administration of liposomal DOP-ddC to mice results in a 40-fold increased retention relative to ddC in the disease target (liver) without a significant increase in the toxicity target (sciatic nerve). We conclude that phospholipid prodrugs of antiviral nucleosides may represent a novel approach to improving efficacy and reducing the toxicity of antiviral agents used in treating HBV. Further studies in animal models of hepatitis B will be needed to evaluate this possibility.

## Acknowledgements

These studies were supported in part by NIH Grant GM-24979 and by the Research Programs of the San Diego Veterans Affairs Medical Center (KYH) and by NIAID grant Contract NO1-AI-72623 (BEK). The authors recognize the excellent technical assistance of Judy Tsuji, Lena Barshak and Richard Border, Vical Inc., San Diego, CA and Kris Wright and Kathy Aldern, University of California, San Diego.

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