ELSEVIER

Antiviral Research 34 (1997) 131–136

Antiviral Research

Lack of mitochondrial toxicity in CEM cells treated with carbovir

William B. Parker [a,*], S.C. Shaddix [a], Robert Vince [b], L. Lee Bennett Jr. [a]

[a] *Kettering–Meyer Laboratory, Southern Research Institute, 2000 Ninth Avenue South, Birmingham AL 35205, USA*
[b] *Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis MN 55455, USA*

Received 2 November 1996; received in revised form 21 January 1997; accepted 21 January 1997

Abstract

Carbovir (CBV) is a guanine nucleoside analog with potent in vitro anti-HIV activity. A prodrug of CBV is currently being evaluated in clinical trials as a potential agent for the treatment of AIDS. The ability of CBV to inhibit mitochondrial DNA synthesis in intact CEM cells was evaluated in the present study, because most of the currently available anti-HIV nucleoside analogs have significant toxicities that result from their inhibition of mitochondrial DNA synthesis. No delayed cytotoxicity was observed in CEM cells treated with 50 μM CBV for 4 weeks. In addition, CBV at concentrations as high as 1 mM did not cause a decline in mitochondrial DNA levels and only minimally increased the concentration of lactic acid in the medium. In contrast to these results with CBV, treatment of CEM cells with 0.5 μM 2′,3′-dideoxycytidine resulted in delayed cytotoxicity, a decrease in mitochondrial DNA content and increases in lactic acid levels in the medium. These results indicated that treatment of CEM cells with CBV did not result in the inhibition of mitochondrial DNA synthesis and suggested that treatment of AIDS patients with CBV, or a prodrug of CBV, would not result in some of the toxicities seen with the other anti-HIV nucleoside analogs. © 1997 Elsevier Science B.V.

Keywords: Carbovir; Mitochondrial DNA synthesis; 1592U89; Lactic acid; Delayed cytotoxicity

1. Introduction

Currently, there are nine drugs available for the treatment of HIV infection. Five of these agents are nucleoside analogs: 3′-deoxy-3′-azidothymidine (AZT), 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxy-2′,3′-didehydrothymidine (D4T) and 2′,3′-dideoxy-

Abbreviations: CBV, carbovir; ddC, 2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; AZT, 3′-deoxy-3′-azidothymidine; D4T, 2′,3′-dideoxy-2′,3′-didehydrothymidine; 3TC, 2′,3′-dideoxy-3′-thiacytidine; CBV-MP, 5′-monophosphate of CBV; CBV-TP, 5′-triphosphate of CBV; 3TC-TP, 5′-triphosphate of 3TC; ddCTP, 5′-triphosphate of ddC; IC_{50}, concentration of drug required to inhibit cell growth by 50%.

* Corresponding author. Fax: +1 205 5812797; e-mail: Parker@SRI.ORG

PII S0166-3542(97)01033-4

3′-thiacytidine (3TC). The other four agents are protease inhibitors. Combination of nucleoside analogs and protease inhibitors have recently shown very impressive anti-HIV activity in patients. Treatment with AZT, ddI, ddC or D4T can result in serious dose-limiting toxicities. The peripheral neuropathy associated with the use of ddI, ddC and D4T and the myopathy associated with the use of AZT are believed to result from the inhibition of DNA polymerase γ, the mitochondrial DNA polymerase [1–4]. The 5′-triphosphates of these nucleoside analogs are all potent inhibitors of DNA polymerase γ [5–8] with K_is for inhibition of DNA polymerase γ that are similar to their K_is for inhibition of HIV-reverse transcriptase. 3TC does not inhibit mitochondrial DNA synthesis, presumably due to the lack of uptake of the triphosphate of 3TC (3TC-TP) by the mitochondria [7]. Because of this lack of selectivity with most of the existing anti-HIV nucleoside analogs, there is need to identify new anti-HIV nucleoside analogs that do not inhibit DNA polymerase γ. Such compounds could be used in place of the existing therapies or could be used in combination with the existing agents to diminish toxicities without diminishing their anti-HIV activity.

Carbovir (CBV, the (-)-enantiomer of the carbocyclic analog of 2′,3′-dideoxy-2′,3′-didehydroguanosine) a guanine nucleoside analog of novel structure, is a potent inhibitor of HIV replication that may be sufficiently different from existing anti-HIV nucleoside analogs in its toxicity and chemical stability to warrant evaluation as a potential anti-AIDS drug [9–12]. CBV inhibits HIV replication in a manner similar to that of the other anti-HIV nucleoside analogs. It is phosphorylated by human enzymes to CBV triphosphate (CBV-TP) [13–16], which is utilized by HIV-RT as a substrate for DNA synthesis using either RNA or DNA as template [17–20]. Because there is no 3′-hydroxyl group, the incorporation of CBV-MP into the DNA chain results in chain termination [18]. We [17,18] and others [21] have shown that CBV-TP is not a potent inhibitor of DNA polymerase γ (K_i of 20 μM). In this study we have evaluated the effect of CBV on mitochondrial DNA synthesis in intact CEM cells to verify the results obtained with isolated enzyme and to determine the potential for the development of mitochondrial toxicities after its use. We found that prolonged incubation of CEM cells with 50 μM CBV did not inhibit mitochondrial DNA synthesis, which suggested that treatment with CBV may not cause peripheral neuropathy or myopathy in patients.

2. Materials and methods

2.1. Cell studies

CEM cells, obtained from the American Type Culture Collection (Rockville, MD) were grown in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) containing 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1 mg/ml sodium bicarbonate, 10 U/ml penicillin, 10 μg/ml streptomycin and 50 μg/ml gentamycin. Cells were routinely tested for the presence of mycoplasmas. New cultures were established from cryopreserved, mycoplasma-free cell stocks. Cells were tested for mycoplasma using Gen-Probe mycoplasmal detection system (Fischer Scientific, Pittsburgh, PA) and by measuring [^{3}H]thymidine uptake [22]. Cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. All experiments were conducted with cells that were proliferating at maximal rates. Cell numbers were determined with a coulter counter. The (-)-enantiomer of CBV was synthesized as previously described [10]. ddC was obtained from Sigma (St. Louis, MO).

2.2. Measurement of mitochondrial DNA content

A mitochondrial DNA probe was made as described by Higuchi and Linn [23] and labeled with [^{32}P]dCTP (ICN Pharmaceuticals, Costa Mesa, CA) using Ready-To-Go DNA labelling beads as described in the manufacturer's instructions (Pharmacia Biotech, Piscataway, NJ). One hundred thousand cells were applied to Zeta-Probe GT blotting membrane (BioRad, Hercules, CA) using a slot blot apparatus after they had been boiled for 10 min in a solution of 0.4 M NaOH

and 10 mM EDTA. Hybridization of the labeled mitochondrial DNA probe was done as described in the manufacturer's instructions, except that the DNA was fixed to the membrane using a GS gene linker UV chamber (BioRad, Hercules, CA) instead of vacuum drying. The amount of probe hybridized to mitochondrial DNA was quantitated both by densitometry of the image on the film and by cutting the dot blots from the membrane and counting them for radioactivity using a Packard Liquid Scintillation Counter. Quantification by these two methods gave similar results.

2.3. Measurement of lactic acid level activity

Lactic acid levels were measured in the medium of cells treated with CBV and ddC using a kit supplied by Boerhinger Mannheim (Indianapolis, IN). In this assay, samples were incubated with lactate dehydrogenase and glutamate-pyruvate transaminase and the increase in NADH was measured spectrophotometrically. The amount of NADH formed in the above reaction is stoichiometric with the amount of lactic acid that is in the sample.

3. Results

CEM cells were incubated with CBV for 72 h to determine the concentration of drug which would inhibit cell growth by 50% (IC_{50}). Under these conditions the IC_{50} of CBV was found to be approximately 100 μM, which was in agreement with previously published results [9,15]. CBV, at the maximal concentration that did not cause acute cytotoxicity (50 μM), did not inhibit CEM cell growth even after 4 weeks of treatment (Fig. 1). As a positive control, CEM cells were also incubated with 0.5 μM ddC. As can be seen in Fig. 1, ddC did not affect cell growth until after 4 days of treatment, but CEM cell growth was totally inhibited by day 11. The time required for the development of delayed toxicity was dependent on the concentration of ddC. These results with ddC support previous work that demonstrated the delayed toxicity of ddC [1].

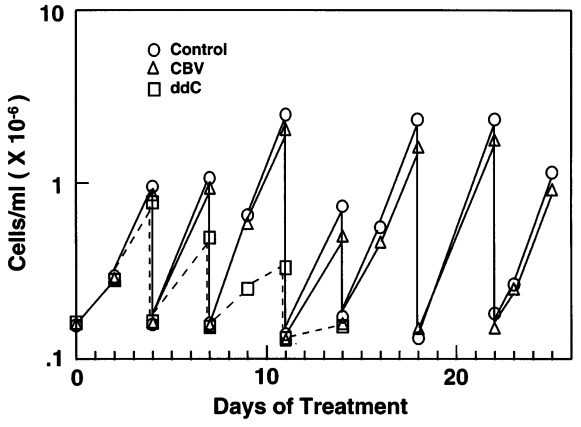

Fig. 1. Effect of CBV and ddC on CEM cell growth. CEM cells were incubated with 50 μM CBV, 0.5 μM ddC, or no drugs and cell numbers were counted regularly for 4 weeks. Cell cultures were diluted in fresh medium containing the appropriate drug on days 4, 7, 11, 14, 18 and 22 to approximately 100 000 cells/ml. The ddC cultures were terminated on day 14 due to lack of growth.

The amount of mitochondrial DNA was determined in CEM cells treated with either 0.5 μM ddC or 50 μM CBV. Measurements were taken throughout the 4 week period. After 9 days of treatment with ddC, mitochondrial DNA levels were decreased by more than 80%, whereas treatment with 50 μM CBV for 9 days had no effect on mitochondrial DNA levels (Fig. 2). The decline in mitochondrial DNA levels in cells treated with ddC was detected as early as 2 days after beginning of treatment and steadily declined thereafter. CBV did not effect of the amount of mitochondrial DNA in CEM cells even after 4 weeks of treatment (data not shown). CEM cells

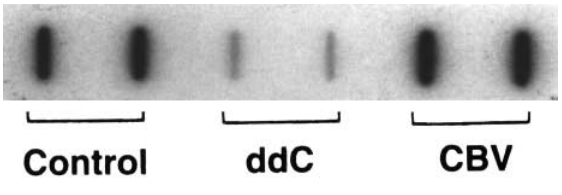

Fig. 2. Effect of CBV and ddC on mitochondrial DNA levels. CEM cells were incubated with 0.5 μM ddC, 50 μM CBV, or no drugs for 9 days with changes in medium on days 4 and 7 as shown in Fig. 1. One hundred thousand cells were removed from each culture, and the mitochondrial DNA levels were determined as described in Section 2. The experiment was repeated one time and similar results were obtained.

Table 1
Lack of effect of CBV on lactic acid levels

Lactic acid per 10^6 cells (mg)			
Day	Control	ddC	CBV
0	0.57	0.53	0.54
4	0.62	0.97	0.70
7	0.46	1.25	0.50
11	0.44	1.60	0.51
14	0.54	0.51	0.55
18	0.45	—	0.47
22	0.43	—	0.44
25	0.41	—	0.47

Cells were incubated with 0.5 μM ddC, 50 μM CBV, or no compound. A sample of media was removed at the times indicated and the concentration of lactic acid was determined. Each number is the average of two separate determinations. Note that the cells were diluted in fresh medium containing the appropriate drug to approximately 100 000 cells/ml on days 4, 7, 11, 14, 18 and 22 after the start of the experiment. Therefore, each measurement shown (other than at day 0) reflects the lactic acid levels in the medium 3 or 4 days after the medium had been changed. The effect of drug treatment on cell growth can be seen in Fig. 1. The experiment was repeated and similar results were obtained.

were also incubated with 1 mM CBV for 96 h and even though CBV caused considerable inhibition of cell growth at this concentration, the amount of mitochondrial DNA per cell was not changed (data not shown).

Medium was collected from CEM cells treated with either 0.5 μM ddC or 50 μM CBV and the lactic acid levels were determined as another indication of mitochondrial toxicity. As can be seen in Table 1, treatment of CEM cells with CBV for 25 days did not cause any increase in the concentration of lactic acid in the medium, whereas, treatment with ddC resulted in three-fold increase in lactic acid levels. At day 14 in ddC-treated cells, the lactic acid levels were similar to those in both control and CBV-treated cells, which was likely due to the fact that these cells were dead (Fig. 1) and were not metabolically active. Treatment of CEM cells with higher concentrations of CBV for 96 h had at best a small effect on lactic acid levels (data not shown). For instance, incubation of CEM cells with either 300 or 1000 μM CBV resulted in lactic acid levels that were only 30 or 50%, respectively, above control levels when corrected for cell number. Because these concentrations were acutely toxic, resulting in 70 or 90% inhibition of cell growth, respectively, it is not known whether these small increases in lactic acid levels are due to inhibition of mitochondrial synthesis or due to some other action of CBV, such as its inhibition of nuclear DNA synthesis [15,18]. Because the DNA sequences of many mitochondrial proteins are coded on nuclear chromosomes, inhibition of chromosomal DNA synthesis could result in mitochondrial toxicity. Regardless, it is clear that treatment with CBV had minimal effect on lactic acid levels.

4. Discussion

The results presented in this work indicated that treatment with CBV has little, if any, effect on mitochondrial DNA synthesis in CEM cells. These data support the results with isolated enzymes that show that CBV-TP is a relatively poor inhibitor of the DNA polymerase γ (CBV-TP is 100–1000-fold less potent as an inhibitor of DNA polymerase γ than is ddCTP and other dideoxynucleotide analogs). We show that incubation with 1 mM CBV for 96 h did not inhibit mitochondrial DNA synthesis even though cell growth was inhibited by about 90%. This concentration of CBV is higher than peak concentrations of CBV in the plasma of animals after either IV or oral administration [24,25]. In addition, the effective dose of CBV against HIV in CEM cells is approximately 0.3 μM [9]. Since millimolar concentrations of CBV would not be maintained in patients being treated for AIDS, these results suggest that it is unlikely that mitochondrial DNA synthesis would be inhibited in patients that receive CBV for treatment of AIDS. However, it is still important to confirm these results in relevant tissues of patients treated with drug.

The lack of activity of CBV against mitochondrial DNA is likely due to the poor phosphorylation of this agent coupled with the relatively high K_i for inhibition of DNA polymerase γ by CBV-TP. We have previously shown that incubation of

CEM cells with 100 μM CBV results in approximately 3 pmol CBV-TP/10^6 cells [15], which is approximately 3 μM intracellular concentration, whereas, the K_i for inhibition of DNA polymerase γ by CBV-TP was 23 μM [18].

Recently, a clinical trial has begun to evaluate a prodrug of CBV as an anti-HIV agent, (1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol (1592U89). This agent has been shown to be one of the most efficacious of the nucleoside analogs when given as a single agent [26,27]. Treatment of AIDS patients for 4 weeks with 1592U89 decreased viral load by approximately 2-logs and caused a 25% increase in CD4 counts. Like CBV, 1592U89 is converted to CBV-TP in CEM cells in culture and this metabolite is believed to be responsible for the pharmacological actions of this agent [21]. Because the active form of both CBV and 1592U89 is CBV-TP, our studies with CBV suggest that little mitochondrial toxicity would be expected after treatment of AIDS patients with 1592U89.

References

[1] Chen, C.-H. and Cheng, Y.-C. (1989) Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency virus compound 2′,3′-dideoxycytidine. J. Biol. Chem. 264, 11 934–11 937.

[2] Medina, D.J., Tsai, C.-H., Hsiung, G.D. and Cheng, Y.-C. (1994) Comparison of mitochondrial morphology, mitochondrial DNA content, and cell viability in cultured cells treated with three anti-human immunodeficiency virus dideoxynucleosides. Antimicrob. Agents Chemother. 38, 1824–1828.

[3] Dalakas, M.C., Illa, I., Pezeshkpour, G.H., Laukaitis, J.P., Cohen, B. and Griffin, J.L. (1990) Mitochondrial myopathy caused by long-term zidovudine therapy. New Engl. J. Med. 322, 1098–1105.

[4] Parker, W.B. and Cheng, Y.-C. (1994) Mitochondrial toxicity of antiviral nucleoside analogs. J. NIH Res. 6, 57–61.

[5] Ono, K., Nakane, H., Herdewijn, P., Balzarini, J. and De Clercq, E. (1989) Differential inhibitory effects of several pyrimidine 2′,3′-dideoxynucleoside 5′-triphosphates on the activities of reverse transcriptase and various cellular DNA polymerases. Mol. Pharmacol. 35, 578–583.

[6] Starnes, M.C. and Cheng, Y.-C. (1987) Cellular metabolism of 2′,3′-dideoxycytidine, a compound active against human immunodeficiency virus in vitro. J. Biol. Chem. 262, 988–991.

[7] Chang, C.-N., Skalskii, V., Zhou, J.H. and Cheng, Y.-C. (1992) Biochemical pharmacology of (+)- and (-)-2′,3′-dideoxy-3′-thiacytidine as anti-hepatitis B virus agents. J. Biol. Chem. 267, 22 414–22 420.

[8] Hart, G.J., Orr, D.C., Penn, C.R., Figueiredo, H.T., Gray, N.M., Boehme, R.E. and Cameron, J.M. (1992) Effects of (-)-2′-deoxy-3′-thiacytidine (3TC) 5′-triphosphate on human immunodeficiency virus reverse transcriptase and mammalian DNA polymerases alpha, beta and gamma. Antimicrobial Agents Chemother. 36, 1688–1694.

[9] Vince, R., Hua, M., Brownell, J., Daluge, S., Lee, F., Shannon, W.M., Lavelle, G.C., Qualls, J., Weislow, O.S., Kiser, R., Canonico, P.G., Schultz, R.H., Narayanan, V.L., Mayo, J.G., Shoemaker, R.H. and Boyd, M.R. (1988) Potent and selective activity of a new carbocyclic nucleoside analog (carbovir: NSC 614846) against human immunodeficiency virus in vitro. Biochem. Biophys. Res. Commun. 156, 1046–1053.

[10] Vince, R. and Brownell, J. (1990) Resolution of racemic carbovir and selective inhibition of human immunodeficiency virus by the (-) enantiomer. Biochem. Biophys. Res. Commun. 168, 912–916.

[11] Carter, S.G., Kessler, J.A. and Rankin C.D. (1990) Activities of (-)-carbovir and 3′-azido-3′-deoxythymidine against human immunodeficiency virus in vitro. Antimicrob. Agents Chemother. 34, 1297–1300.

[12] Coates, J.A.V., Inggall, H.J., Pearson, B.A., Penn, C.R. Storer, R., Williamson, C. and Cameron, J.M. (1991) Carbovir: the (-) enantiomer is a potent and selective antiviral agent against human immunodeficiency virus in vitro. Antiviral Res. 15, 161–168.

[13] Johnson, M.A. and A. Fridland.(1989) Phosphorylation of 2′,3′-dideoxyinosine by cytosolic 5′-nucleotidase of human lymphoid cells. Mol. Pharmacol. 36, 291–295.

[14] Bondoc, L.L., Jr., Shannon, W.M., Secrist, III, J.A., Vince, R. and Fridland, A. (1990) Metabolism of the carbocyclic nucleoside analogue carbovir, an inhibitor of human immunodeficiency virus, in human lymphoid cells. Biochem. 29, 9839–9843.

[15] Parker, W.B., Shaddix, S.C., Bowdon, B.J., Vince, R. Shannon, W.M. and Bennett, Jr., L.L. (1993) Metabolism of carbovir, a potent inhibitor of human immunodeficiency virus type 1 replication, and its effect on cellular metabolism. Antimicrob. Agents Chemother. 37, 1004–1009.

[16] Miller, W. H., Daluge, S.M., Garvey, E.P., Hopkins, S. Reardon, J.E., Boyd, F.L. and Miller, R.L. (1992) Phosphorylation of carbovir enantiomers by cellular enzymes determines the stereoselectively of antiviral activity. J. Biol. Chem. 267, 21 220–21 224.

[17] White, E.L., Parker, W.B., Macy, L.J., Shaddix, S.C., McCaleb, G., Secrist, III, J.A., Vince, R. and Shannon, W.M. (1989) Comparison of the effect of carbovir, AZT, and dideoxynucleoside triphosphates on the activity of

human immunodeficiency virus reverse transcriptase and selected human polymerases. Biochem. Biophys. Res. Commun. 161, 393–398.

[18] Parker, W.B., White, E.L., Shaddix, S.C., Ross, L.J., Buckheit Jr., R.W., Germany, J.M., Secrist III, J.A., Vince, R. and Shannon, W.M. (1991) Mechanism of inhibition of human immunodeficiency virus type I reverse transcriptase and human DNA polymerases α, β, and γ by the 5′-triphosphates of carbovir, 3′-azido-3′-deoxythymidine, 2′,3′-dideoxyguanosine and 3′-deoxythymidine. A novel RNA template for the evaluation of antiretroviral drugs. J. Biol. Chem. 266, 1754–1762.

[19] Miller, W. H., Daluge, S.M., Garvey, E.P., Hopkins, S. Reardon, J.E., Boyd, F.L. and Miller, R.L. (1992) Phosphorylation of carbovir enantiomers by cellular enzymes determines the stereoselectively of antiviral activity. J. Biol. Chem. 267, 21 220–21 224.

[20] Orr, D.C., Figueiredo, H.T., Mo, C.-L., Penn, C.R. and Cameron, J.M. (1992) DNA chain termination activity and inhibition of human immunodeficiency virus reverse transcriptase by carbocyclic 2′,3′-didehydro-2′,3′-dideoxyguanosine triphosphate. J. Biol. Chem. 267, 4177–4182.

[21] Faletto, M.B., Miller, W.H., Garvey, E.P., St. Clair, M.H., Hazen, R.J., Daluge, S.M. and Good, S.S. (1995) Unique purine crossover activation pathway for the potent anti- HIV agent 1592U89. Eighth Int. Conf. Antiviral Res. 26, A262.

[22] Merkenschlager, M., Kardamakis, D., Rawle, F.C., Spurr, N. and Beverly, P.C.L. (1988) Rate of incorporation of radiolabelled nucleosides does not necessarily reflect the metabolic state of cells in culture: effects of latent mycoplasma contamination. Immunology 63, 125–131.

[23] Higuchi, Y. and Linn, S. (1995) Purification of all forms of HeLa cell mitochondrial DNA and assessment of damage to it caused by hydrogen peroxide treatment of mitochondria or cells. J. Biol. Chem. 270, 7950–7956.

[24] Yeom, Y.-H., Remmel, R.P., Huang, S.-H., Hua, M., Vince, R. and Zimmerman, C.L. (1989) Pharmacokinetics and bioavailability of carbovir, a carbocyclic nucleoside active against human immunodeficiency virus, in rats. Antimicrob. Agents Chemother. 33, 171–175.

[25] El Dareer, S.M., Tillery, K.F., Rose, L.M., Struck, R.F. and Hill, D.L. (1990) Disposition and metabolism of carbovir in mice dosed intravenously or orally. Drug Metabolism Disposition 18, 842–845.

[26] Saag, M., Lancaster, D., Sonnerborg, A., Mulder, J., Torres, R., Schooley, R., Harrigan, R., Kelleher, D. and Symonds, W. (1996) A phase I/II study of a novel nucleoside reverse transcriptase inhibitor, 1592U89 monotherapy vs. zidovudine (ZVD) or placebo in HIV infected patients with CD4 counts 200–500/mm^3. 3rd Conf. Retroviruses Opportunistic Infections, 89.

[27] Hughes, W., Mcdowell, J., Adams, L., Flynn, P., Hetherington, S., Kline, M., Shenep, J., Yogev, R. and Lafon, S. (1996) Evaluation of the novel nucleoside 1592U89 in a phase I safety and pharmacokinetics (PK) study in HIV-infected infants and children. 3rd Conf. Retroviruses Opportunistic Infections, 115.