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Some nucleoside analogs with anti-human immunodeficiency virus activity inhibit replication of Epstein–Barr virus

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Abstract

The effects of (+)-β-D-dioxolane-cytosine ((+)-D-β-DOC), (−)-β-L-dioxolane-cytosine ((−)-L-β-DOC), (+)-β-D-oxathiolane-cytosine ((+)-D-β-OTC), (−)-β-L-oxathiolane-cytosine ((−)-L-β-OTC, or 3TC), 3′-azido-2′,3′-dideoxy-5-methyl-cytidine (5-Me-AZDC), and 3′-azido-2′,3′-dideoxyuridine (AZDU) on Epstein–Barr virus (EBV) DNA replication in vitro were tested in P3HR-1 cells. Two anti-EBV drugs, 3′-azido-3′-deoxythymidine (AZT) and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, or ganciclovir), were used as positive controls. The inhibitory effects on EBV DNA synthesis were quantified by membrane filter and Southern blot hybridizations with an EBV-specific probe *Bam*HI-W fragment. The 50% effective doses (ED_{50}) for EBV DNA replication were 0.15, 0.83, 1.5, 8.3, 14, and 7.7 μM for DHPG, (−)-L-β-DOC, (+)-D-β-DOC, (+)-D-β-OTC, (−)-L-β-OTC, and AZT, respectively. In contrast, 5-Me-AZDC and AZDU were not effective at concentrations as high as 30 μM. These results indicated that both (−)-L-β-DOC and (+)-D-β-DOC were more potent than AZT, which has previously been shown to have anti-EBV activity. (−)-L-β-DOC and (+)-D-β-DOC have also been previously demonstrated to suppress the infectivity of human immunodeficiency virus type 1 (HIV-1). Thus, (−)-L-β-DOC represents the first nucleoside analog with L-configuration exhibiting significant antiviral activities against both EBV and HIV.

Keywords: Nucleoside analog;Epstein–Barr virus, Inhibition

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1. Introduction

Epstein–Barr virus (EBV), a ubiquitous human lymphotropic virus, is the causative agent for infectious mononucleosis (IM) (Henle et al., 1968), associated with African Burkitt's lymphoma (Ziegler, 1981), nasopharyngeal carcinoma, T-cell lymphoma, Hodgkin's lymphoma, and non-Hodgkin's lymphoma in immunocompromised individuals (Miller, 1990). Like other herpesviruses (Davis et al., 1987; Mosca et al., 1987), EBV upregulates transcription of human immunodeficiency virus (HIV) through activation of its 5′-long terminal repeat (Kenney et al., 1988; Mallon et al., 1990; Lin, 1993). The interaction between EBV and HIV at the molecular level may have a biological significance. For instance, some studies indicate that the disease in those infected with a second virus progresses more rapidly than in those infected with HIV alone (Nelson et al., 1990). Additionally, it has been previously demonstrated that HIV and EBV can co-infect B-cells (Casareale et al., 1983; Montagnier et al., 1984; Tozzi et al., 1989).

Patients with AIDS have a high incidence of EBV-related B-cell lymphomas including Burkitt's lymphoma (Ziegler et al., 1982). Furthermore, the data from clinical trials showed that HIV- infected patients treated with AZT and acyclovir (ACV) have longer survival rates than those treated with AZT alone (Cooper et al., 1993), implicating that herpesviruses may act as 'co-factors' in HIV infection and stimulate the spread of HIV in the body. These results prompted us to search for agents that can simultaneously inhibit both HIV and herpesviruses, alleviating the complication of opportunistic viral infections and thus improving the quality of life of AIDS patients.

In our search for antiherpetic compounds, we discovered that an anti-HIV drug, AZT, strongly inhibited EBV replication (Lin et al., 1988). Inhibition was selective and unique in that the drug showed no detectable effects on replication of other human herpesviruses, cytomegalovirus, herpes simplex virus types 1 and 2, and varicella-zoster virus. Recently, a series of AZT derivatives (Chu et al., 1988; Schinazi et al., 1990) and novel nucleoside analogs with modification at the ribosyl ring (Kim et al., 1992) were synthesized and evaluated against EBV DNA replication. In this study we wish to report preliminary results of anti-EBV activities of some representative compounds which have previously been shown to have anti-HIV activity.

2. Materials and methods

2.1. Cell cultures

A virus-producer cell line (P3HR-1 cells) was propagated in RPMI 1600 medium containing 8% heat-inactivated fetal calf serum. Cells were maintained in exponential growth as described previously (Lin et al., 1982).

2.2. Treatment of cells with drugs

To enhance the sensitivity of detection of the drug effects, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, 30 ng/ml) and sodium butyrate (Na butyrate, 3 mM) were added

to the exponentially growing cells (5×10^7 cells/25 cm^2-flask), together with the testing drugs at various concentrations. After 4 days of treatment, cells were replenished with fresh medium containing TPA and testing drugs. Cells were incubated for another 3 days before being processed for DNA isolation. Three independent experiments were performed, and triplicate samples were processed for each experiment.

2.3. DNA isolation

Control and drug-treated cells were pelleted and washed with cold phosphate-buffered solution, followed by treatment with proteinase K (150 μg/ml) in buffer A containing 50 mM Tris-HCl, pH 9.0, 0.5 M NaCl, 10 mM EDTA and 1% sodium lauryl sarcosine at 55°C for 14 h. The resultant mixture was extracted with phenol and then chloroform. After precipitation with cold ethanol, DNA was resuspended in 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA.

2.4. Southern blot hybridization

Total cellular DNAs digested with *Bam*HI restriction enzyme were electrophoresed on 1% agarose gel and Southern blotted onto a nitrocellulose sheet. After blotting, the DNA was UV cross-linked by a UV Stratalinker (Stratagene, La Jolla, CA), and baked in a vacuum oven at 80°C for 30 min. The sheet was hybridized with an EBV-specific *Bam*HI-W fragment probe.

2.5. Determination of EBV genome copy numbers

Approximately 20 μg of total cellular DNA was immobilized on the nitrocellulose membrane and hybridized with an EBV-specific *Bam*HI-W fragment, which contains the large internal repeated sequence within the EBV genome. The probe was prepared by labeling DNA with [α-^{32}P]dCTP (ICN, Irvine, CA) and random-primer kit (Stratagene, La Jolla, CA). The conditions at which filter membrane hybridization was carried out have been described (Smiley et al., 1988) and EBV genome copy numbers were determined using Raji cellular DNA as a standard reference of 50 copies per cell (Lin et al., 1984). A reconstruction of standard curve with different amounts of Raji DNA is routinely performed in parallel with the testing samples. In addition, for each batch of Raji DNA, we calibrate EBV genome copy numbers in Raji cells using cloned *Bam*HI-W DNA.

2.6. Determination of ID_{50} for cell growth

To measure the effects of drugs on cell growth in terms of ID_{50} (the concentration of a drug at which the cell growth is inhibited by 50%), cells were treated with various concentrations of testing drugs without TPA, ranging from 10 to 250 μM. Cell numbers were counted daily and cell viability was determined by trypan blue exclusion method.

2.7. Chemicals

(+)-β-D-Dioxolane-cytosine ((+)-D-β-DOC), (−)-β-L-dioxolane-cytosine ((−)-L-β-DOC), (+)-β-D-oxathiolane-cytosine ((+)-D-β-OTC), (−)-β-L-oxathiolane-cytosine ((−)-L-β-OTC, or 3TC), 3′-azido-2′,3′-dideoxy-5-methyl-cytidine (5-Me-AZDC), and 3′-azido-2′,3′-dideoxyuridine (AZDU) were synthesized as reported previously (Chu et

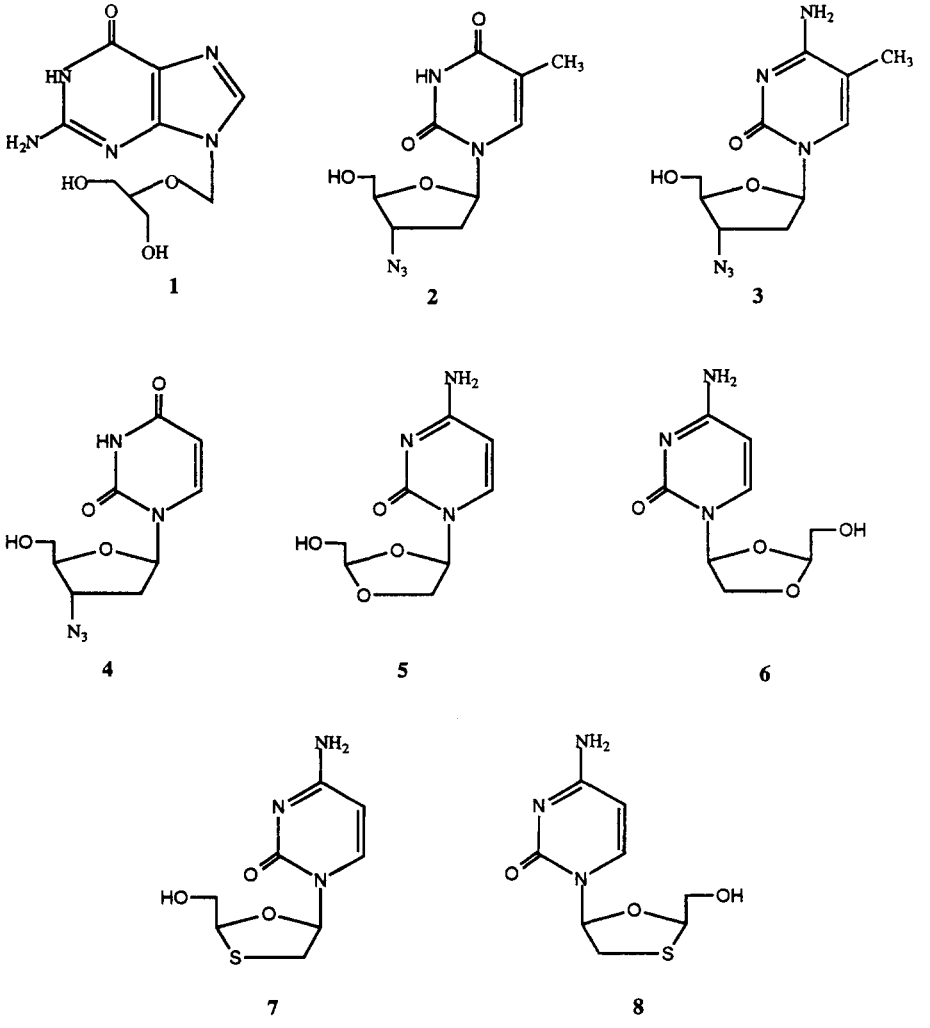

Fig. 1. Chemical structures of nucleoside analogs. **1**, 9-(1,3-Dihydroxy-2-propoxymethyl)guanine (DHPG); **2**, 3′-azido-3′-deoxythymidine (AZT); **3**, 3′-azido-3′-deoxy-5-methyl-cytidine (5-Me-AZDC); **4**, 3′-azido-2′,3′-dideoxyuridine (AZDU); **5**, (+)-β-D-dioxolane-cytosine ((+)-D-β-DOC); **6**, (−)-β-L-dioxolane-cytosine ((−)-L-β-DOC); **7**, (+)-β-D-oxathiolane-cytosine ((+)-D-β-OTC); **8**, (−)-β-L-oxathiolane-cytosine ((−)-L-β-OTC, or 3TC).

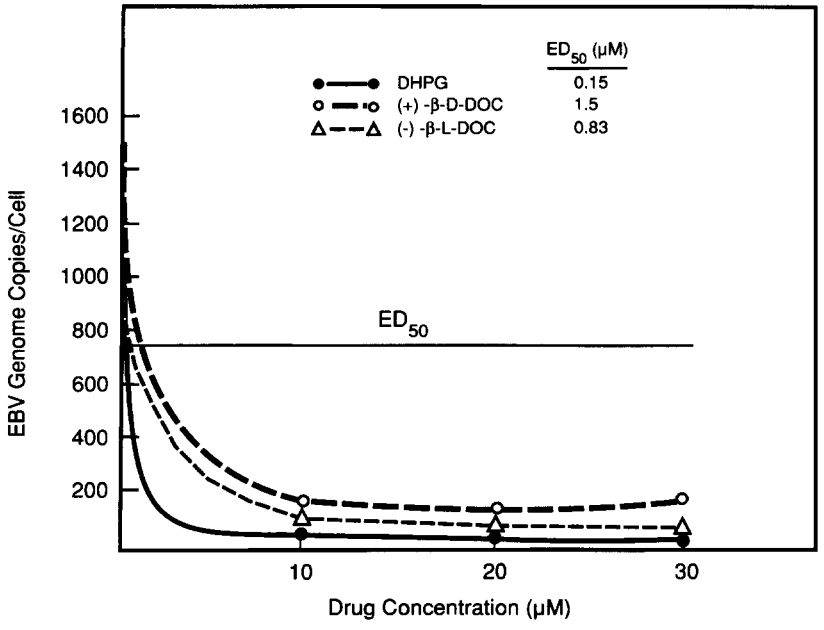

Fig. 2. Determination of viral ED_{50} of DHPG, (+)-D-β-DOC, and (−)-L-β-DOC. Exponentially growing P3HR-1 cells were seeded at a density of 3×10^6 per ml and incubated in medium containing TPA/Na butyrate and various concentrations of drugs for 7 days. EBV genome copy numbers per cell determined at each drug concentration are the average of 3 determinations.

al., 1988; Schinazi et al., 1990; Kim et al., 1992). 3′-Azido-3′-deoxythymidine (AZT) and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, or ganciclovir) were a gift from Burroughs Wellcome Co., Research Triangle Park, NC.

3. Results

Fig. 1 shows the chemical structures of nucleoside analogs used for this study. Numbers **1** and **2** are positive control compounds, DHPG and AZT, respectively. Numbers **3–8** are the testing new compounds with modifications at the ribosyl ring.

Based on the structural similarity, the testing results were plotted in 3 groups. Fig. 2 shows the dose-dependent effects of EBV DNA replication by (+)-D-β-DOC and (−)-L-β-DOC. EBV genome copy numbers per cell decreased with increasing drug concentrations in both cases but at different rates. The treatment effectively eradicated the linear form of EBV DNA and left the 30 copies of episomal DNA intact (Lin et al., 1984). The complete elimination of linear form of EBV DNA requires continued treatment for at least 14 days (Lin et al., 1984). The episomal DNA is not affected by any nucleoside analogs tested so far (data not shown). Assuming that the residual genome level (30 copies per cell) achieved by the effective drug DHPG is the zero point and that the viral genome level without drug treatment (1500 copies/cell) is 100, ED_{50}

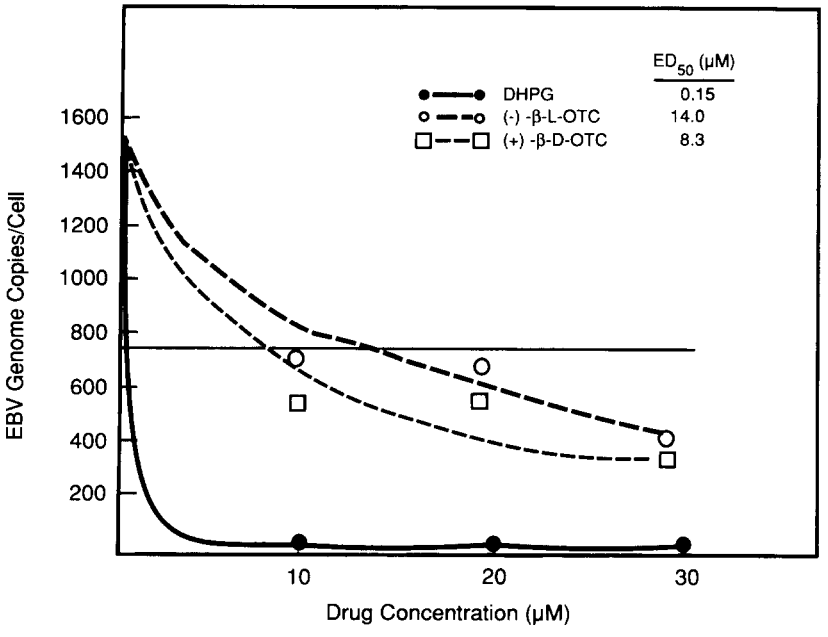

Fig. 3. Determination of viral ED_{50} of DHPG, (+)-D-β-OTC, and (−)-L-β-OTC. The conditions for drug treatment and the determination of EBV genome copy numbers are the same as in Fig. 2.

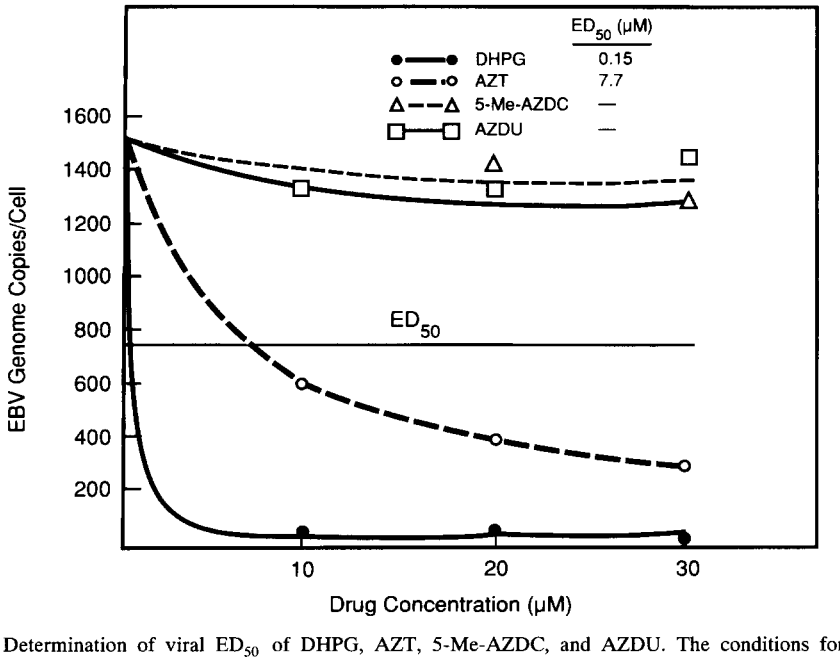

Fig. 4. Determination of viral ED_{50} of DHPG, AZT, 5-Me-AZDC, and AZDU. The conditions for drug treatment and the determination of EBV genome copy numbers are the same as in Fig. 2.

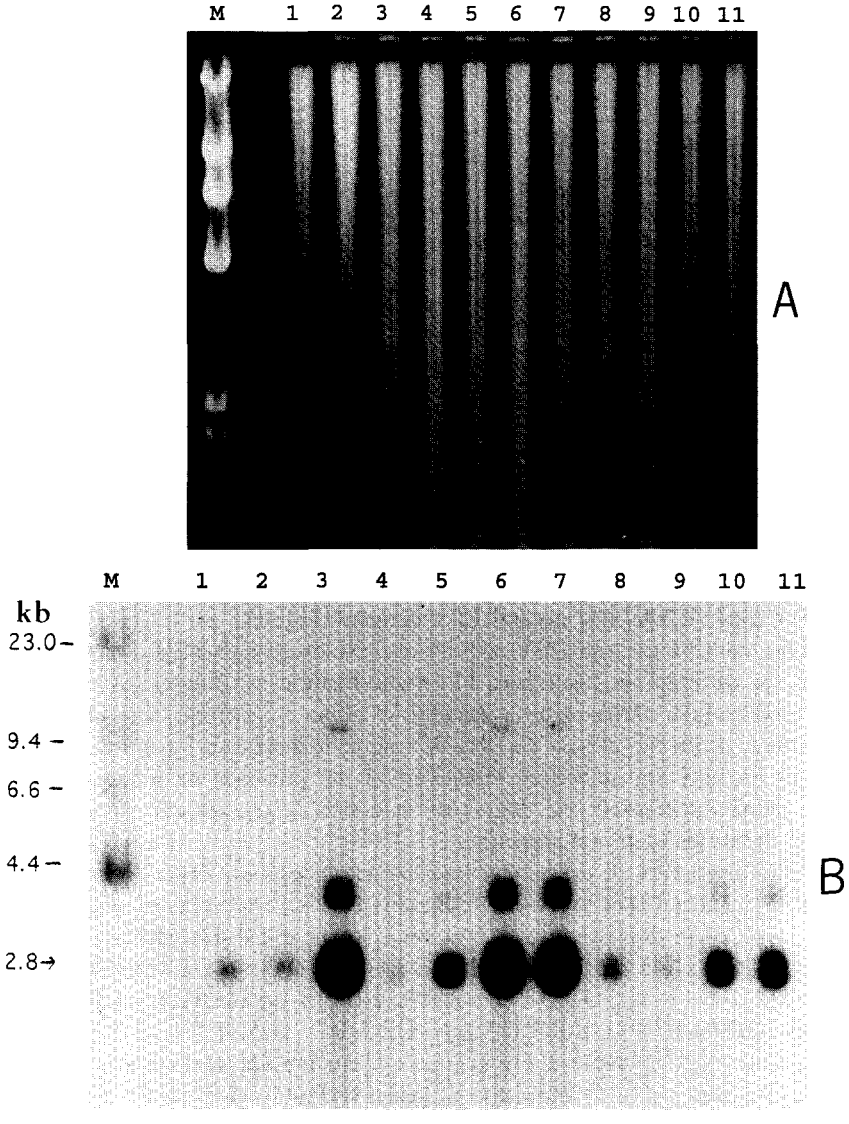

Fig. 5.

represents the mid-point between 1500 and 30 (Lin et al., 1984). From the plot we determined that the ED_{50}s for viral inhibition were 0.15, 1.5, and 0.83 μM for DHPG, (+)-D-β-DOC, and (−)-L-β-DOC, respectively. The same graphical method was used to determine the ID_{50}s for cell growth. On the basis of these data, we calculated the therapeutic index (ID_{50}/ED_{50}).

The testing results with (+)-D-β-OTC and (−)-L-β-OTC are shown in Fig. 3. Again, similar dose-dependent inhibition of EBV DNA replication was observed with these two

Table 1
Inhibitory effect of nucleoside analogs on EBV DNA replication in P3HR-1 cells

Drug	ED_{50} (μM)	ID_{50} (μM)	Therapeutic index (ID_{50}/ED_{50})
1 DHPG	0.15	200	1333
2 AZT	7.7 (0.004) [a]	200	26
3 5-Me-AZDC	> 30 (0.15)	ND [b]	ND
4 AZDU	> 30 (0.32)	ND	ND
5 (+)-D-β-DOC	1.5 (0.016)	150	100
6 (−)-L-β-DOC	0.83 (0.002)	30	36
7 (+)-D-β-OTC	8.3 (0.21)	30	3.6
8 (−)-L-β-OTC (3TC)	14 (0.0018)	10	0.7

[a] The values in parentheses are ED_{50} values for HIV-1 tested in PBMC (Chu et al., 1989; Kim et al., 1992, 1993; Jeong et al., 1993a,b).
[b] ND, not determined.

drugs, but the inhibitory effects were not as potent as with (+)-D-β-DOC and (−)-L-β-DOC. The ED_{50} values were 8.3 and 14 μM for (+)-D-β-OTC and (−)-L-β-OTC, respectively.

Although AZT is active against EBV replication, however, two AZT derivatives, 5-Me-AZDC and AZDU, had no anti-EBV activity (Fig. 4).

To further confirm the anti-EBV activity of these drugs measured by membrane filter hybridization, we employed Southern blot hybridization of *Bam*HI restriction enzyme digested total cellular DNA using a *Bam*HI-W clone as a probe. The intensity of the signals derived from the 2.8-kb *Bam*HI-W fragment increased by more than 50 times after TPA/Na butyrate induction. However, in the presence of inducers and testing drugs the signals decreased; the degree of reduction in signals corresponded with the potency of the drugs (data not shown).

The inhibitory action of these new nucleoside analogs on EBV DNA replication is summarized in Table 1. For purpose of comparison, the ED_{50} value of each drug against HIV-1 is included in the parentheses. The relative anti-EBV potency of these drugs, as compared to DHPG and AZT, was: DHPG > (−)-L-β-DOC > (+)-D-β-DOC > AZT > (+)-D-β-OTC > (−)-L- β-OTC. The relative efficacy on the basis of the therapeutic index was: DHPG > (+)-D-β-DOC > (−)-L-β-DOC > AZT > (+)-D-β-OTC > (−)-L-β-OTC. Thus, among these newly synthesized nucleoside analogs tested, the derivatives with dioxalane, both D- and L-form, appear to be the most potent EBV inhibitors.

4. Discussion

In this study we demonstrated that (+)-D-β-DOC, (−)-L-β-DOC, (+)-D-β-OTC, and (−)-L-β-OTC are potent inhibitors of EBV DNA synthesis. However, (+)-D-β-DOC and (−)-L-β-DOC are more potent and have higher therapeutic indices than their thio-derivatives, (+)-D-β-OTC and (−)-L-β-OTC, against EBV DNA replication. The anti-EBV activities of these nucleoside analogs reported in this study appeared to be selective in that they inhibited virus replication at drug concentrations which were not cytotoxic or cytostatic in vitro. The 50% inhibitory doses for viral replication (ED_{50}s)

were far below their 50% inhibitory doses for cell growth (see Table 1). In addition, within the dose ranges tested, they exhibited cytostatic effects at higher drug concentrations, but not cytotoxic effects. It should be noted that the L-form isomers were 3- to 5-fold more cytostatic than their corresponding D-form isomers.

The differences in the inhibitory effects between dioxolane- and oxathiolane-derivatives could, in part, be due to a variation in the efficiency of drug phosphorylation by cellular and/or viral nucleoside kinase(s), or in the incorporation of the drug triphosphate into the viral template by EBV DNA polymerase. Further studies on the intracellular phosphorylation of these nucleosides and the interactions of the phosphorylated forms of the drugs with EBV DNA polymerase may be required, which is in progress in our laboratories. Recently, we have successfully cloned and functionally expressed the EBV DNA polymerase gene both in vitro (Lin et al., 1991) and in the baculovirus system (Lin et al., 1994). The availability of a large quantity of purified EBV DNA polymerase will facilitate this study.

(−)-L-β-OTC (3TC), the enantiomer of (+)-D-β-isomer of BCH-189 (Kim et al., 1992), is currently undergoing clinical trials in Europe, Canada, and the United States, both as an anti-HIV and anti-HBV agent. This (−)-enantiomer (L-form) is more potent than the (+)-enantiomer (D-form) and is resistant to cytidine/deoxycytidine deaminase (Schinazi et al., 1992). BCH-189 has also been shown to be a potent inhibitor of human hepatitis B virus (HBV) in cell culture (Doong et al., 1991). It appears that the L-form (3TC) of BCH-189 is a more potent inhibitor against HIV-1 and HBV in vitro. However, in this study both (+)-D-β-OTC (D-form) and (−)-L-β-OTC (L-form) showed moderate activity against EBV replication. Whether L-isomers of dioxolane- and oxathiolane-derivatives are more efficacious in terms of antiviral activities in general requires further investigation.

Previous observations indicated that AZT exhibits both anti-EBV and HIV activities. In the present study, we discovered that both DOC and OTC also possess dual antiviral activities, with the L-form being the most potent against HIV-1 (Table 1). Unlike AZT, structurally (−)-L-β-DOC is an L-form nucleoside analog which rarely occurs in nature. Despite this, (−)-L-β-DOC is approximately 9-fold more potent than AZT against EBV and has a better therapeutic index in vitro. In addition, (−)-L-β-DOC is 2-fold more potent than AZT and 8-fold more potent than (+)-D-β-DOC in inhibiting HIV-1 replication in vitro (Table 1). The unexpected finding of these drugs with dual antiviral actions provides a lead for further development of this series of compounds which may be of benefit to AIDS patients with opportunistic herpesviral infections.

Acknowledgements

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