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The SATE pronucleotide approach applied to acyclovir Part II. Effects of bis(SATE)phosphotriester derivatives of acyclovir on duck hepatitis B virus replication in vitro and in vivo

Olivier Hantz [a,*], Christian Périgaud [b,1], Christelle Borel [a], Catherine Jamard [a], Fabien Zoulim [a], Christian Trépo [a], Jean-Louis Imbach [b], Gilles Gosselin [b]

[a] *I.N.S.E.R.M. U. 271, 151 cours Albert Thomas, 69424 Lyon Cedex 03, France*
[b] *Laboratoire de Chimie Bioorganique, U.M.R. C.N.R.S. 5625, Case Courrier 008, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France*

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Abstract

The in vitro and in vivo antiviral activities of two mononucleoside phosphotriester derivatives of acyclovir (ACV) incorporating *S*-acyl-2-thioethyl (SATE) groups are reported using the duck model of hepatitis B (DHBV). In primary duck hepatocyte cultures, the described phosphotriesters significantly inhibited the replication of DHBV at submicromolar concentrations. They were found to be more potent than the parent nucleoside. This result was in agreement with our data concerning the anti-HBV activity of these pronucleotides in HepG2.2.15 cells (previous paper). In vivo, the studied SATE pronucleotide was also found to be more efficient than ACV in infected ducklings upon short-term oral therapy, while intraperitoneal treatment showed high anti-DHBV activity with both ACV and its SATE pronucleotide in this animal model. These findings demonstrate the potential of SATE pronucleotides of ACV as anti-HBV agents.

Keywords: Acyclovir; Duck Hepatitis B virus; Nucleotide; Prodrug

1. Introduction

Hepatitis B virus (HBV) infection remains a major public health problem with 300 million chronic carriers world-wide, leading to severe liver diseases, cirrhosis and hepatocellular carcinoma.

* Corresponding author for U. 271. Tel.: +33-4-726-81982; fax: +33-4-726-81971; e-mail: hantz@lyon151.inserm.fr.
[1] Corresponding author for U.M.R. 5625. Tel.: +33-4-67144776; fax: +33-4-67042029; e-mail: perigaud@univ-montp2.fr

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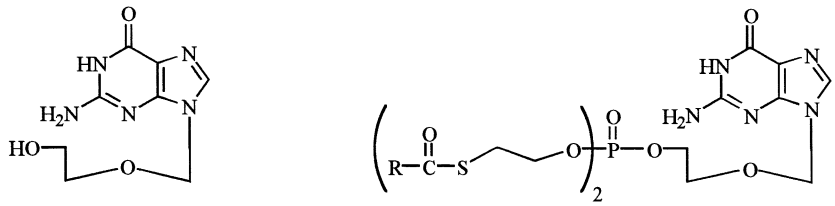

ACV

1 bis(MeSATE)ACVMP, R= CH_3
2 bis(tBuSATE)ACVMP, R= $(CH_3)_3C$

Fig. 1. Structure of ACV and its corresponding bis(SATE) phosphotriester derivatives.

Currently, interferon α is the most widely used therapy against HBV infection but is effective in only 30–40% of selected patients, with a much lower response rate in long-term HBV carriers (Hantz et al., 1993; Hoofnagle and Dibisceglie, 1997). More potent antiviral drugs for the treatment of hepatitis B are necessary, but the search has been complicated by the restricted host range of HBV. The most susceptible animal, the chimpanzee, develops acute and persistent HBV infection, but this model is unsuitable for routine confirmation of the anti-HBV activity of compounds that are active in vitro.

Fortunately, the replication of HBV-like viruses in other animal species provides alternatives in smaller and more amenable models. Human HBV is the prototype member of the Hepadnavirus family (Gust et al., 1986), which includes very closely related viruses in ducks, woodchucks and ground squirrels. The duck virus (DHBV or duck hepatitis B virus) model has been largely used for the screening and the evaluation of new anti-HBV agents both in vitro in primary duck hepatocyte cultures (Fourel et al., 1989; Bishop et al., 1990; Yokota et al., 1990; Heijtink et al., 1994) and in vivo in chronically infected animals (Fourel et al., 1992, 1994; Mason et al., 1994; Zoulim et al., 1996).

Acyclovir (ACV, Fig. 1), an acyclic guanosine analogue, is well known as an anti-herpetic agent (O'Brien and Campoli-Richards, 1989; Wagstaff et al., 1994). ACV is preferentially phosphorylated by herpes-encoded thymidine kinase to give ACV 5′-monophosphate (ACVMP), which is then converted to ACV 5′-triphosphate (ACVTP) by cellular kinases. ACVTP has a greater affinity for viral DNA polymerases than for cellular DNA polymerases and is incorporated as a chain terminator into the nascent viral DNA. ACVTP also inhibits in vitro the human and woodchuck HBV DNA polymerases (Hantz et al., 1984) and the DHBV reverse transcriptase activity (Dannaoui et al., 1997), although its parent nucleoside ACV has limited activity against HBV in vivo (Weller et al., 1982; Guarascio et al., 1986). This is likely due to the lack of HBV encoded thymidine kinase which should catalyze the first phosphorylation step.

We have previously reported (Périgaud et al., 1999) that the dependence of ACV for the first phosphorylation step could be circumvented by the use of mononucleotide prodrugs (pronucleotides). Thus, two bis(SATE) phosphotriester derivatives of ACV [bis(MeSATE)ACVMP and bis(tBuSATE)ACVMP, Fig. 1] have been found to exhibit potent and selective in vitro anti-HBV activity in human hepatoblastoma-derived liver Hep-G2 cells (2.2.15 cells). The aim of the present study was to evaluate the efficacy of these pronucleotides in both primary duck hepatocyte cultures and in vivo experimentally infected ducklings.

2. Materials and methods

2.1. Starting materials and chemical synthesis

The synthesis and physico-chemical properties of the test compounds **1** and **2** have been previously reported (Périgaud et al., 1999).

2.2. In vitro studies

2.2.1. Liver perfusion and hepatocyte primary cultures

Three-week old ducklings chronically infected by DHBV were used for the preparation of fresh hepatocytes. The ducks were killed under anesthesia and the isolation of the hepatocytes was performed by in situ two-step collagenase perfusion of the liver (Turin et al., 1996). At the end of the perfusion, the cell suspension was filtered through gauze (60 μm pore size) and enriched by three differential centrifugations. Cells were seeded on six-well plates at a density of 5×10^5 per well in Leibowitz medium supplemented with 5 μg per ml of bovine insulin, 7×10^{-5} M hydrocortisone hemisuccinate and 10% FCS. One day after cell plating, medium was changed with the same medium supplemented with 1.5% DMSO (Pugh and Summers, 1989) instead of FCS. The compounds that were tested for antiviral activity were added to the culture 2 days after plating during 9 days (three wells per assay). Medium was changed every day and saved for further testing.

2.2.2. Analysis of viral DNA and IC_{50} calculation

DHBV DNA was detected in the hepatocytes culture supernatants or in duck sera by a DNA-spot hybridization as previously described (Lambert et al., 1991). Briefly, 800 μl of culture medium or 50 μl of serum was spotted directly onto nitrocellulose filters using a BRL hybridot manifold apparatus (Gibco-BRL SARL Cergy-Pontoise, France). After denaturation and neutralization, filters were hybridized with a full length cloned DHBV DNA probe obtained by random-priming labelling of full length DNA insert purified from plasmid by Eco R1 digestion and agarose gel electrophoresis. After autoradiography, the individual spots were counted in a scintillation counter. IC_{50} was defined as the effective drug concentration that induces a 50% decrease in the level of DHBV DNA in culture supernatants and calculated by linear regression analysis. Data from three independent experiments were combined.

Protein-bound (viral DNA intermediate) and non-protein-bound (CCC for covalently closed circular viral DNA) intracellular DNA were separated by selective precipitation of SDS-complexed protein with KCl, as described (Summers et al., 1990). Total intracellular DNA was prepared as previously described (Lambert et al., 1991). Briefly, after incubation of the cells suspended in TNE solution with proteinase K in the presence of 0.1% SDS, proteins were removed by extraction with phenol and chloroform. Nucleic acids were precipitated with ethanol and stored at −20°C. DNA was electrophoresed, transferred to nitrocellulose and hybridized with DHBV DNA probe.

2.2.3. Cytotoxicity

Cytotoxicity measurements were based on the estimation of hepatocytes viability after drug treatment using uptake of neutral red dye as described previously (Fautz et al., 1991). Primary duck hepatocytes were seeded at a density of 5×10^4 cells per well in 24-well tissue culture plates. After plating, cells were cultivated in 1 ml of medium containing various concentrations of the test compound with daily changes. Four wells per assay were used. After 9 days of treatment, cell viability was estimated by a neutral red (with a 0.005% neutral red solution) uptake method as determined by absorbance at 540 nm. The minimum cytotoxic concentration or CC_{50} was defined as the concentration required to reduce cell viability by 50%.

2.3. In vivo studies

2.3.1. Experimental animals

French Pekin ducks (*Anas platyrhynchos*) were obtained from a commercial breeder and housed in our colony. Ducklings were inoculated intravenously with a standard DHBV inoculum containing 1.5×10^7 viral genome equivalents 2 days post hatching as described previously (Lambert et al., 1991).

2.3.2. Drug treatment

Five ducklings in each treatment group were treated 3 days postinoculation with ACV, bis(t-BuSATE)phosphotriester derivative **2** in dimethyl sulfoxide solution (2 mg/ml) or placebo either by

Table 1
Effect of the bis(SATE)phosphotriester derivatives of ACV **1** and **2** compared to their nucleoside parent ACV against DHBV replication in hepatocytes primary culture

Compound	IC_{50}[a](μM)	CC_{50}[b](μM)	Selectivity index (CC_{50}/IC_{50})
1	0.03 ± 0.01	5	166
2	0.0006 ± 0.003	120	200 000
ACV	0.1 ± 0.02	>2000	$>20\,000$

[a] IC_{50}, drug concentration that induces a 50% decrease in the level of DHBV replication, as based on the inhibition of extracellular DHBV DNA after 9 days of treatment. Values are mean of three independent experiments.
[b] CC_{50}, cytotoxic concentration or concentration required to reduce cell viability by 50%, as based on the viability of cells after 9 days of treatment by a neutral red assay.

oral route or by intraperitoneal injection for 5 days according to the protocol described in Section 3. Blood samples were taken daily before, during therapy and for 7 days after the end of treatment. The duckling weight was monitored daily. On cessation of treatment, one duck in each group was sacrificed under anesthesia and livers were harvested and stored at -70°C for further analysis.

3. Results and discussion

The use of pronucleotides represents an attractive approach to improve the therapeutic potential of nucleoside analogues and in the last few decades, many strategies have been devised (Alexander and Holý, 1994; Krise and Stella, 1996; Périgaud et al., 1996a). The SATE groups have emerged as very efficient transient phosphate protection of 5′-mononucleotide analogues in cell culture experiments (Périgaud et al., 1997). For example, the SATE prodrug concept has been applied to antiviral nucleoside analogues hampered at the first activation step by a dependence on kinase-mediated phosphorylation (Girardet et al., 1995) or by a rate limiting step later in the anabolic pathway (Périgaud et al., 1994), and to acyclic nucleoside phosphonates having difficulty in crossing cell membranes due to their polar nature (Benzaria et al., 1996). In all cases, the use of the corresponding SATE pronucleotides led in vitro to an increase in the antiviral activity of the parent nucleosides or phosphonate (Périgaud et al., 1996b). Furthermore, the SATE pronucleotide approach extends the antiviral spectrum of the antiherpetic agent ACV, since the bis(SATE)-phosphotriester derivatives of ACV showed potent and selective anti-HBV activity in 2.2.15 cells (Périgaud et al., 1999).

Compared to permanently transfected cell lines, primary duck hepatocytes provide an in vitro system in which the complete viral cycle replication occurs. Moreover, DHBV infection serves as an available animal model system for evaluating antiviral activity in vivo. In this study, the evaluation of two previously synthesized (Périgaud et al., 1999) bis(SATE)phosphotriester derivatives of ACV (Fig. 1) was extended to both duck hepatocyte cultures and experimentally infected ducklings.

3.1. In vitro evaluation of the SATE pronucleotides

The pronucleotides **1** and **2** were evaluated for their inhibitory effects on the replication of DHBV in hepatocyte cultures and compared to ACV. In this assay, the two phosphotriester derivatives significantly inhibited the replication of DHBV at submicromolar concentrations (Table 1). This result was in agreement with our data concerning the anti-HBV activity of these pronucleotides in 2.2.15 cells (Périgaud et al., 1999). Nevertheless, all compounds were more active in duck hepatocyte cultures than in 2.2.15 cells. As compared to cultured hepatoblastoma cells, the greater activity in primary cells, in which a more dynamic replication occurs and high virus yields are achieved, may reflect an increased con-

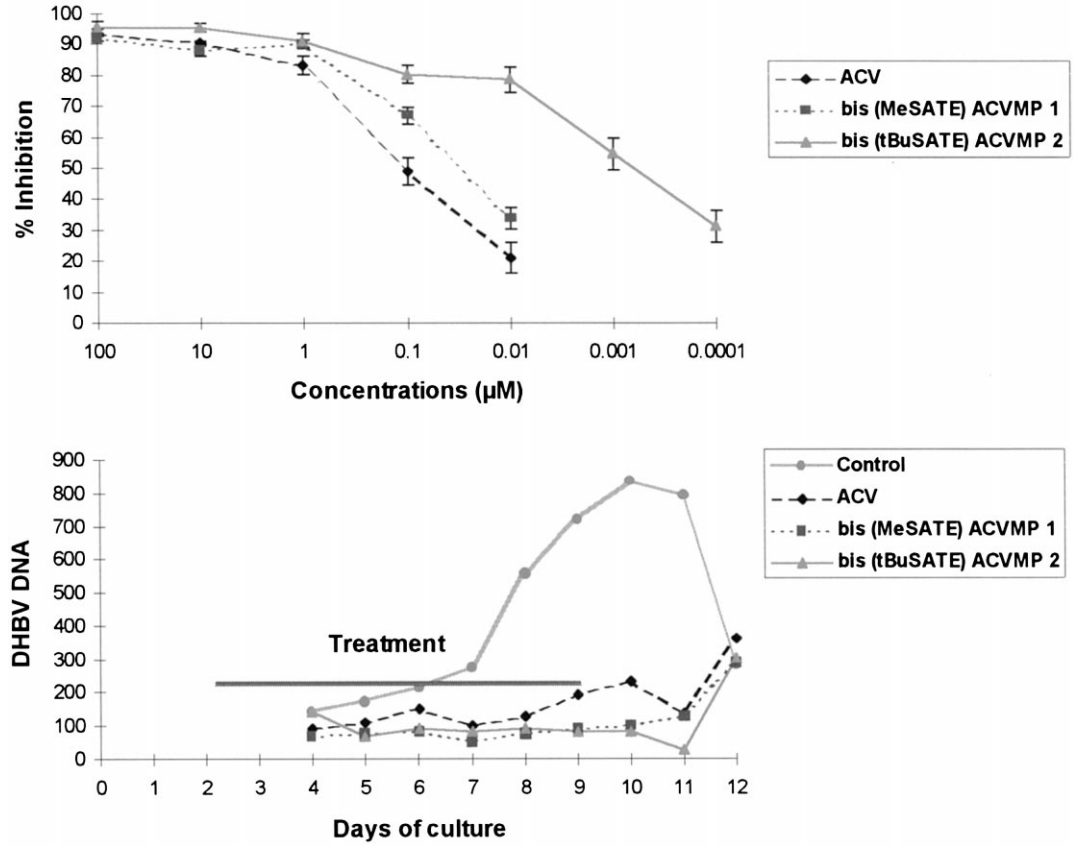

Fig. 2. (A) Dose-dependent inhibition of DHBV in duck primary hepatocyte cultures. Cultures were treated for 9 days with ACV (–◆–), bis(MeSATE)ACVMP **1** (–■–) and bis(tBuSATE)ACVMP **2** (–▲–) at the indicated concentration. DHBV DNA levels in triplicate culture supernatants taken on the last day of treatment were measured by a dot-spot hybridization assay as described in Section 2. Values are the mean of three independent experiments and are expressed as % inhibition with respect to DHBV DNA level in supernatants of control cultures. (B) Changes in DHBV DNA level in culture supernatants of hepatocytes treated for 9 days with 1 μM of ACV (–◆–), bis(MeSATE)ACVMP **1** (–■–) and bis(tBuSATE)ACVMP **2** (–▲–) or untreated (–●–).

version of these compounds to ACVTP. The IC_{50} value obtained for ACV (0.1 μM) is much lower than that reported previously in the same cell cultures (Yokota et al., 1990). These differences may be due to culture conditions and to the antiviral assays used.

All compounds inhibited DHBV replication in duck primary hepatocyte cultures in a dose-dependent manner (Fig. 2A). In marked contrast to the evaluation in 2.2.15 cells, striking differences were found in the antiviral activity of the test compounds **1** and **2** in duck hepatocyte cultures. Factors including the lipophilicity of these compounds and/or their kinetics of decomposition (in culture medium and inside the cell) may be involved in the observed in vitro anti-DHBV activity. More complete and detailed studies will be necessary to investigate the effect of the intracellular delivery of ACVMP on the observed antiviral activity. The bis(tBuSATE)phosphotriester **2** emerged as the most potent inhibitor with a 50% inhibitory concentration (IC_{50}) value of 0.0006 μM, which was about 150-fold higher than that of ACV (Table 1); **2** showed a high selectivity index (SI 200 000). In all cases, withdrawal of the test compounds from the culture medium resulted in a rapid increase of viral replication (Fig. 2B), as frequently observed for anti-HBV nucleoside ana-

logues. Analysis of intracellular viral DNA extracted from cells at the end of treatment showed that all compounds clearly inhibited the formation of replicative intermediates (single-stranded DNA) and that of the relaxed circular (RC) DNA (Fig. 3). Covalently closed circular (CCC) DNA levels in treated culture were diminished but not completely abolished, and thus could serve as a template to reinitiate viral replication after drug withdrawal at day 9 of treatment.

As the primary hepatocytes remained quiescent under the culture conditions used, the antiproliferative effect of the studied compounds could not be determined. The viability of uninfected cells following drug incubation, in conditions similar to that of antiviral assays, was measured by a neutral red assay. Compared to ACV, a significant increase in toxicity was observed with the mononucleoside phosphotriester derivatives **1** and **2** (Table 1). Using clonogenic assays, we have previously demonstrated that the SATE pro-moieties, as well as their degradation products, do not induce additional toxicity in normal human bone marrow progenitor cells (Périgaud et al., 1996c). Consequently, the enhanced toxicity observed for the SATE pronucleotides **1** and **2** may reflect the intracellular accumulation of the corresponding (mono-, di- and tri-) phosphorylated forms of ACV which possibly interact with cellular host enzymes. In keeping with this hypothesis, it was reported that ACVTP could interact with cellular DNA polymerases (Johansson, 1993; Ilsley et al., 1995) and that the selective inhibition of herpes simplex virus replication by ACV largely occurs because monophosphorylation is dependent upon viral-encoded thymidine kinase. This result illustrates that despite the proven efficiency of the SATE pronucleotide approach (Gosselin et al., 1996; Périgaud et al., 1996b), the 5'-triphosphate derivative has to be highly selective against viral DNA polymerase versus host DNA polymerases in order to avoid concomitant cytotoxicity (Périgaud et al., 1996c).

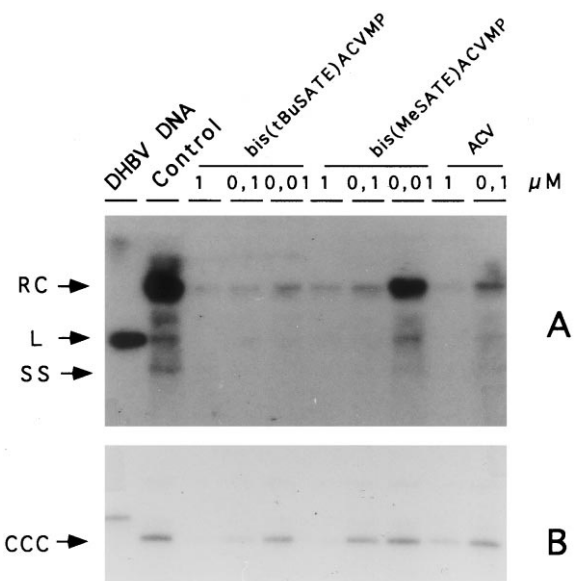

Fig. 3. Southern blot analysis of intracellular DHBV DNA extracted at day 9 from duck hepatocyte cultures untreated or treated with ACV, bis(MeSATE)ACVMP **1** and bis(tBuSATE)-ACVMP **2** at indicated concentration. (A) Protein-bound viral DNA intermediates, relaxed circular (RC), linear (L) and single stranded (SS) (upper panel) were separated from (B) free episomal DHBV cccDNA (lower panel) as described in Section 2.

3.2. In vivo evaluation of the SATE pronucleotides

Given the potent and selective inhibition of DHBV replication in duck hepatocyte cultures of the bis(tBuSATE)phosphotriester derivative **2**, we decided to investigate the efficacy of this pronucleotide, in comparison to ACV, in infected ducklings. It was reported that, administered orally, ACV suppressed the replication and the production of infectious virions during treatment in this animal model (Zuckerman, 1987). This result, in apparent contrast with the failure of ACV treatment in patients with chronic HBV, may be explained by a different hepatic metabolism in avian and mammal cells (Zuckerman, 1987). Moreover, we observed in our in vivo assays that ducklings showed an important gain of weight during the experimental period. Consequently, it is likely that the level of cellular enzymes involved in the phosphorylation of nucleoside analogues is more important in growing liver cells than in quiescent adult liver (Shaw and Locarnini, 1995). In preliminary experiments, ACV or pronucleotide **2** given intraperitonealy (50, 20 then 5 mg/kg per day),

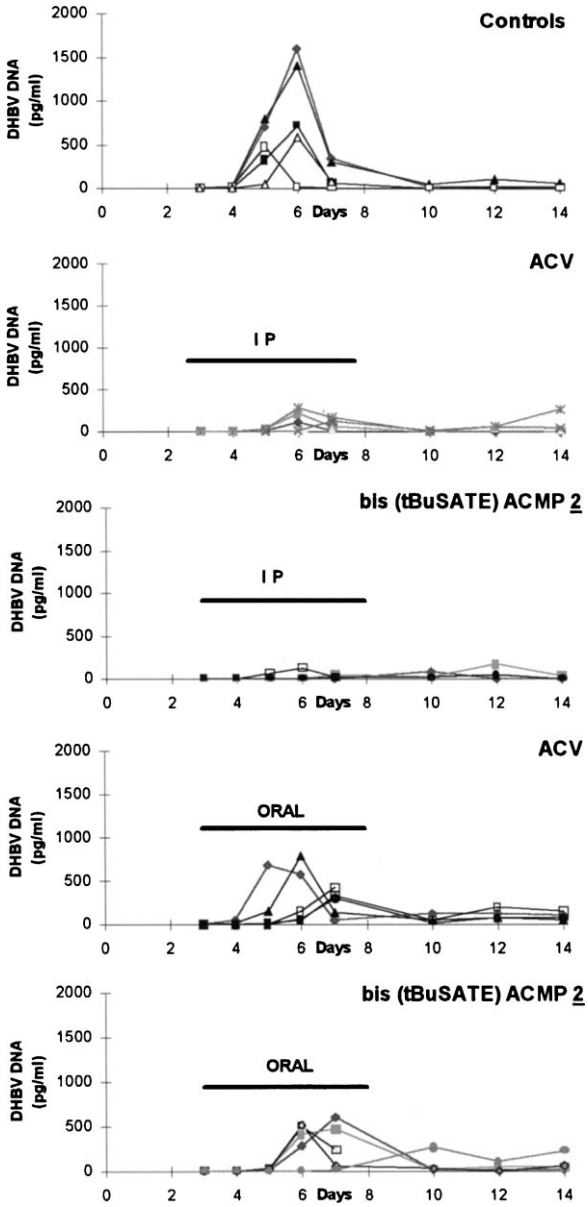

Fig. 4. In vivo antiviral activity of ACV and bis(tBuSATE)-ACVMP **2** in DHBV-infected ducks. Ducklings were inoculated with a standard DHBV inoculum 2 days post hatching and then treated at day 3 post-inoculation for 5 days either by the oral route (10 mg/kg per day) or intraperitoneal route (5 mg/kg per day). Five animals were used in control groups and in each treatment group. DHBV DNA levels in the sera were measured by a dot-blot hybridization assay. The mean level of DHBV DNA in each group is plotted.

induced an almost complete inhibition of viral replication in treated animals (Fig. 4), indicating that both compounds were active in vivo. This result can be explained by the fact that the peritoneal cavity offers a large absorbing surface from which drugs (in dimethyl sulfoxide solution) enter the portal vein rapidly and directly reach the liver. However, this mode of administration is unsuitable for human therapy. Thus, the pronucleotide **2** and ACV were given orally first at high dose (50 mg/kg per day). In such conditions, we observed an almost complete inhibition of DHBV replication during treatment, as measured by the level of viral DNA in the sera of treated animals (data not shown). In a second set of experiments, the two compounds were administrated orally at lower doses, i.e. 10 mg/kg per day orally. Results confirmed the anti-DHBV activity of the pronucleotide **2** (Fig. 4). Given orally at 10 mg/kg per day, both ACV and the bis(tBuSATE)phosphotriester derivative **2** inhibited the viral replication by approximately 50% as measured by the level of circulating DHBV DNA in the sera of treated ducks. Analysis of viral DNA in the duck liver after 5 days of treatment confirmed the partial inhibition of DHBV replication at the dose used. Among the different forms of intracellular viral DNA, the RC and linear DNA were clearly reduced while the CCC DNA was less affected (data not shown). Compared to ACV, the oral administration of pronucleotide **2** produced a similar effect on the viremia peak in treated animals (Fig. 4). However, the molecular weight of this phosphorylated prodrug (593.66 g/mol) is 2.6-fold more elevated than that of ACV (225.21 g/mol). Thus, in terms of molar effective drug concentration, the bis(tBuSATE)phosphotriester derivative **2** proved to be superior to ACV as an anti-DHBV agent after oral administration.

4. Conclusion

In conclusion, using duck hepatocyte primary cultures in which a complete viral life cycle takes place, we confirmed the in vitro potency of SATE pronucleotides of ACV previously demonstrated in human HepG2.2.15 cells (Périgaud et al., 1999). In contrast with its lack of anti-HBV activity in

the human hepatoma cell line, ACV was active against DHBV in duckling hepatocytes but far less than its phosphotriester derivatives **1** and particularly **2**. In vivo, the bis(tBuSATE)-phosphotriester derivative **2** was also superior to ACV after both intraperitoneal and oral administration, even if its superiority was less evident than in vitro. Chronically DHBV-infected ducklings appear to be a particular animal species where ACV, which is poorly active against HBV in human, exhibits a potent antiviral effect. Other factors including aqueous solubility and stability in duck serum of bis(tBuSATE)phosphotriester derivative **2** may be involved in its in vivo anti-DHBV activity (Valette et al., 1997). The high efficiency of intraperitoneal treatment, as compared to the oral route, indicates that improvement of oral bioavailability of SATE pronucleotides could increase their in vivo anti-HBV activity. The present results represent a successful example of the SATE pronucleotide approach applied to acyclovir for the treatment of chronic hepatitis B in an animal model.

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