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The *S*-acyl-2-thioethyl pronucleotide approach applied to acyclovir
Part I. Synthesis and in vitro anti-hepatitis B virus activity of bis(*S*-acyl-2-thioethyl)phosphotriester derivatives of acyclovir☆

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Abstract

The synthesis and in vitro anti-hepatitis B virus (HBV) activity of two mononucleoside phosphotriester derivatives of acyclovir incorporating *S*-acyl-2-thioethyl (SATE) groups are reported. In contrast to the parent nucleoside, the described phosphotriesters emerged as potent and selective inhibitors of HBV replication in HepG2.2.15 cells. This result can be attributed to the unique cellular metabolism of the SATE pronucleotides giving rise to the delivery to acyclovir 5′-monophosphate inside the infected cells. Moreover, the in vitro anti-HBV activities of one of these bis(SATE)phosphotriesters and of (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (lamivudine, 3TC) were compared alone and in combination. Analysis of the combination data indicates that 3TC and the studied SATE pronucleotide of acyclovir exhibited strong synergistic interactions. The present study provides an example where the use of a pronucleotide approach extends the antiviral spectrum of a nucleoside analogue. Given the potency of SATE pronucleotides of acyclovir against HBV in HepG2.2.15 cells, further studies including animal experiments seem warranted to evaluate the potential of these compounds as anti-HBV agents.

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

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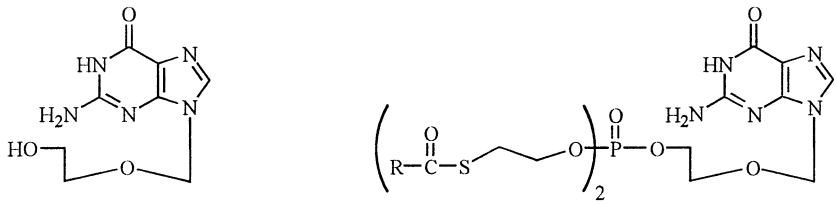

Fig. 1. Structures of ACV and its corresponding bis(SATE)phosphotriester derivatives.

1. Introduction

Acyclovir [9-(2-hydroxyethoxymethyl)guanine, ACV, Zovirax®] is currently used as a therapeutic agent for the treatment of herpes simplex (HSV) and varicella zoster (VZV) virus infections (Wagstaff et al., 1994; O'Brien and Campoli-Richards, 1989). Its selectivity for HSV and VZV is due to its preferential monophosphorylation in the virus-infected cells by a herpesvirus-induced thymidine kinase. The resulting ACV 5′-monophosphate is subsequently converted to the corresponding 5′-triphosphate derivative (ACVTP) by cellular enzymes. ACVTP inhibits herpesvirus DNA polymerase, and its incorporation into viral DNA results in chain termination. ACV remains essentialy unchanged in uninfected cells; consequently, there is little interference with cellular DNA synthesis. Specificity for the herpesvirus-induced thymidine kinase accounts for the wide therapeutic index of ACV but limits its activity spectrum, essentially confined to HSV and VZV and excluding important pathogens such as human hepatitis B virus (HBV) which does not encode a thymidine kinase. Thus, the efficacy of ACV in treating patients with chronic HBV is uncertain (Wagstaff et al., 1994), although ACVTP is a relatively good inhibitor of HBV DNA polymerases (Hantz et al., 1984). Randomised comparative studies of ACV alone (Guarascio et al., 1986; Alexander et al., 1987) or in combination with interferon (Berk et al., 1992) failed to show a statistically significant effect on the rate of seroconversion. These results illustrate the need to develop approaches capable of delivering (and targeting if possible) preformed phosphorylated ACV forms inside the HBV-infected cells and organs. In this respect, our group has recently reported the potential as antiviral agents of neutral mononucleoside phosphotriesters which incorporate biolabile *S*-acyl-2-thioethyl (SATE) phosphate protecting groups (Lefebvre et al., 1995). With 3′-azido-2′,3′-deoxythymidine (AZT) as nucleoside model, it has been demonstrated that the corresponding bis(SATE)phosphotriester derivatives were able to liberate the parent 5′-monophosphate inside the cell through an esterase-mediated activation process (Périgaud et al., 1997). Here, in order to exploit the potency of ACVTP as anti-HBV agent, two bis(SATE)phosphotriester derivatives of ACV [bis(MeSATE)-ACVMP and bis(tBuSATE)ACVMP; Fig. 1] were synthesized and evaluated for their inhibitory effects on the replication of human HBV in HBV DNA-transfected human hepatoblastoma-derived liver Hep-G2 cells (2.2.15 cells). This cell line has been shown to be an accurate model for chronic cellular viral replication as well as a predictive model for the in vivo antiviral response in hepadnaviral infection (Korba and Milman, 1991; Korba and Gerin, 1992).

2. Materials and methods

2.1. Anti-HBV and cytotoxicity assays

Antiviral and toxicity analyses were performed as previously described (Korba and Gerin, 1992). Briefly, confluent cultures of the HBV-producing cell line 2.2.15 in 96-well flat-bottomed tissue culture plates were treated with nine consecutive

daily doses of the test compounds (six cultures for each of four dilutions). Medium was changed daily with fresh test compounds. The levels of HBV virion DNA in the culture medium were determined 24 h following the last treatment by dot blot hybridization using a full-length ^{32}P-labelled HBV DNA fragment probe. HBV DNA was quantitated by comparison to standards included on each blotting membrane using an AMBIS beta scanner.

Cultures were treated with combinations of agents (Korba, 1996). Compounds were mixed at molar ratios designed to give approximately equipotent antiviral effects based on the EC_{90} values for the monotherapies. Serial dilutions of these mixtures were then used to treat cultures as described above along with the appropriate monotherapies except that eight cultures were used for each of six dilutions. Analysis of synergism, additivity, or antagonism were determined by analysis of the data using the CALCUSYN™ program (Biosoft, Inc., Cambridge, UK).

Cells for the toxicity analyses were cultured and treated with test compounds with the same schedule and under identical culture conditions as used for the antiviral evaluations (Korba and Gerin, 1992). Each compound was tested at four concentrations, each in triplicate cultures. Uptake of neutral red dye was used to determine the relative level of toxicity 24 h following the last treatment. The absorbance of internalized dye at 510 nm (A_{510}) was used for quantitative analysis of the treated cultures relative to nine cultures of untreated cells maintained on the same 96-well plate as the test compounds.

2.2. Starting materials and chemical synthesis

ACV was obtained from ISIS Pharmaceuticals (Carlsbad, CA). (−)-β-L-2′,3′-Dideoxy-3′-thiacytidine (lamivudine, 3TC) was purchased from Moravek Biochemicals (Brea, CA USA). N^2-(4-Monomethoxytrityl)-9-(2-hydroxyethoxymethyl) guanine was synthesized according to a published procedure (Martin et al., 1986). The synthesis of bis(*S*-acetyl-2-thioethyl) *N*,*N*-diisopropylphosphoramidite (**3**) and bis(*S*-pivaloyl-2-thioethyl) *N*,*N*-diisopropylphosphoramidite (**4**) have been described already, as were the general procedures and instrumentation used (Lefebvre et al., 1995). High-performance liquid chromatography (HPLC) studies were carried out on a Waters Associates unit equipped with a model 616 pump system, a model 600S system controller, a model 996 photodiode array detector and a Millenium data workstation. The column was a reverse-phase analytical column (Nucleosil, C_{18}, 150 × 4.6 mm, 5 μm) protected by a prefilter and a pre-column (Nucleosil, C_{18}, 5 μm). The pronucleotides to be analyzed were eluted using a linear gradient of 0–80% acetonitrile in 50 mM triethylammonium acetate buffer (pH 7.0) over a 40-min period, with a flow rate of 1 ml/min and detection at 260 nm.

The test compounds were found to be pure by rigorous HPLC analysis, high-field multinuclear nuclear magnetic resonance (NMR) spectroscopy, quantitative ultraviolet (UV) spectra, mass spectroscopy and elemental analysis.

2.2.1. General procedure for the preparation of the phosphotriesters **5** and **6**

1*H*-Tetrazole (210 mg, 3.0 mmol) was added to a stirred solution of N^2-(4-monomethoxytrityl)-9-(2-hydroxyethoxymethyl)guanine (500 mg, 1.0 mmol) and the appropriate phosphoramidite **3** or **4** (1.2 mmol) in tetrahydrofuran (3.0 ml). After 30 min at room temperature, the reaction mixture was cooled to −40°C and a solution of 3-chloroperoxybenzoic acid (407 mg, 1.3 mmol) in methylene chloride (5 ml) was added. The solution was allowed to warm up to room temperature over 1 h. Sodium hydrogen sulfite (10% aqueous solution, 3 ml) was added to reduce the excess of 3-chloroperoxybenzoic acid. The organic layer was separated, diluted with methylene chloride (10 ml), washed with saturated aqueous sodium hydrogen carbonate (3 ml) and then with water (3 × 3 ml), dried over sodium sulfate and evaporated to dryness. The residue was taken up in the minimum amount of methylene chloride and chromatographed on a silica gel column using a stepwise gradient of methanol (0–4%) in methylene chloride. The title compounds **5** and **6** were obtained as white foams.

*2.2.1.1. [N^2-(4-monomethoxytrityl)guanin-9-yl] methyloxy-2-ethyl bis(S-acetyl-2-thioethyl) phosphate (**5**).*

Yield, 531 mg (68%). ^{1}H NMR (DMSO-d_6): δ 10.63 (s, 1H, NH), 7.71 (s, 1H, NH), 7.68 (s, 1H, 8-H), 7.3–7.1 (m, 12H, aromatic), 6.86 (d, 2H, aromatic, $J = 8.9$ Hz), 4.86 (s, 2H, NCH_2O), 3.97 (m, 4H, OCH_2CH_2S), 3.7 (bs, 5H, OCH_3 and $POCH_2CH_2O$), 3.10 (t, 4H, OCH_2CH_2 S, $J = 6.3$ Hz), 3.1–3.0 (m, 2H, $POCH_2CH_2O$), 2.35 (s, 6H, CH_3COS). ^{31}P NMR (DMSO-d_6): δ −0.82. Mass spectra (glycerol–thioglycerol 1:1, v/v), FAB > 0 m/z: 782 $[M+H]^+$, 152 $[BH_2]^+$, 103 $[CH_3COSCH_2CH_2]^+$; FAB < 0 m/z: 1561 [2M-H]$^-$, 780 [M-H]$^-$, 678 [M-$CH_3COSCH_2CH_2$]$^-$, 576 [M-2($CH_3COSCH_2CH_2$) + H]$^-$, 301 [($CH_3COSCH_2CH_2O$)$_2PO_2$]$^-$, 199 [$CH_3COSCH_2CH_2OPO_3$H]$^-$, 150 [B]$^-$.

*2.2.1.2. [N^2-(4-monomethoxytrityl)guanin-9-yl] methyloxy-2-ethyl bis(S-pivaloyl-2-thioethyl) phosphate (**6**).*

Yield, 615 mg (71%). ^{1}H NMR (DMSO-d_6): δ 10.63 (s, 1H, NH), 7.71 (s, 1H, NH), 7.67 (s, 1H, 8-H), 7.3–7.1 (m, 12H, aromatic), 6.86 (d, 2H, aromatic, $J = 8.9$ Hz), 4.87 (s, 2H, NCH_2O), 3.97 (m, 4H, OCH_2CH_2S), 3.7 (bs, 5H, OCH_3 and $POCH_2CH_2O$), 3.09 (t, 4H, OCH_2CH_2S, $J = 6.4$ Hz), 3.1–3.0 (m, 2H, $POCH_2CH_2O$), 1.17 (s, 18H, $(CH_3)_3COS$). ^{31}P NMR (DMSO-d_6): δ −0.87. Mass spectra (glycerol–thioglycerol 1:1, v/v), FAB > 0 m/z: 866 $[M+H]^+$, 145 $[(CH_3)_3CCOSCH_2CH_2]^+$; FAB < 0 m/z: 864 [M-H]$^-$, 720 [M-$(CH_3)_3CCOSCH_2CH_2$]$^-$, 385 [$((CH_3)_3CCOSCH_2CH_2O)_2PO_2$]$^-$, 241 [$(CH_3)_3CCOSCH_2CH_2OPO_3$H)$^-$.

*2.2.2. General procedure for the preparation of the ACV pronucleotides **1** and **2***

A solution of the appropriate phosphotriester **5** or **6** (0.5 mmol) in a mixture of acetic acid, methanol and water (35 ml, 8:1:1, v/v) was heated at 50°C for 18 h and then evaporated to dryness. The residue was coevaporated three times with ethanol and twice with methylene chloride. The residue was taken up in a minimum amount of methylene chloride and chromatographed on a silica gel column, using a gradient of methanol (0–10%) in methylene chloride, to give the pure title compounds **1** and **2**.

*2.2.2.1. Guanin-9-yl methyloxy-2-ethyl bis(S-acetyl-2-thioethyl) phosphate (**1**).*

Yield, 235 mg (92%). UV: λ_{max} (EtOH 95) 253 nm (ε 14100). ^{1}H NMR (DMSO-d_6): δ 10.65 (s, 1H, NH), 7.81 (s, 1H, 8-H), 6.5 (bs, 2H, NH_2), 5.35 (s, 2H, NCH_2O), 4.1–3.9 (m, 6H, $POC\underline{H}_2CH_2O$ and $OC\underline{H}_2CH_2S$), 3.7–3.6 (m, 2H, $POCH_2C\underline{H}_2O$), 3.09 (t, 4H, $OCH_2C\underline{H}_2S$, $J = 6.3$ Hz), 2.34 (s, 6H, CH_3COS). ^{31}P NMR (DMSO-d_6): δ −0.74. Mass spectra (glycerol–thioglycerol 1:1, v/v), FAB > 0 m/z: 510 $[M+H]^+$, 408 [M-$CH_3COSCH_2CH_2$ + 2H]$^+$, 152 $[BH_2]^+$, 103 $[CH_3COSCH_2CH_2]^+$; FAB < 0 m/z: 1018 [2M-H]$^-$, 406 [M-$CH_3COSCH_2CH_2$]$^-$, 304 [M-2($CH_3COSCH_2CH_2$) + H]$^-$, 301 [($CH_3COSCH_2CH_2O$)$_2PO_2$]$^-$, 199 [$CH_3COSCH_2CH_2OPO_3$H]$^-$, 150 [B]$^-$. HPLC t_R 17.3 min. Anal. Calcd for $C_{16}H_{24}N_5O_8PS_2$: C, 37.72; H, 4.75; found: C, 37.85; H, 4.97.

*2.2.2.2. Guanin-9-yl methyloxy-2-ethyl bis(S-pivaloyl-2-thioethyl) phosphate (**2**).*

Yield, 252 mg (85%). UV: λ_{max} (EtOH 95) 253 nm (ε 13400). ^{1}H NMR (DMSO-d_6): δ 10.59 (s, 1H, NH), 7.79 (s, 1H, 8-H), 6.5 (bs, 2H, NH_2), 5.36 (s, 2H, NCH_2O), 4.1–4.0 (m, 6H, $POC\underline{H}_2CH_2O$ and $OC\underline{H}_2CH_2S$), 3.7–3.6 (m, 2H, $POCH_2C\underline{H}_2O$), 3.08 (t, 4H, $OCH_2C\underline{H}_2S$, $J = 6.3$ Hz), 1.17 (s, 18H, $(CH_3)_3C$). ^{31}P NMR (DMSO-d_6): δ −0.79. Mass spectra (glycerol–thioglycerol 1:1, v/v), FAB > 0: m/z 594 $[M+H]^+$, 152 $[BH_2]^+$, 145 $[(CH_3)_3CCOSCH_2CH_2]^+$; FAB < 0: m/z 592 [M-H]$^-$, 448 [M-$(CH_3)_3CCOSCH_2CH_2$]$^-$, 385 [$((CH_3)_3CCOSCH_2CH_2O)_2PO_2$]$^-$, 241 [$(CH_3)_3CCOSCH_2CH_2OPO_3$H]$^-$, 150 [B]$^-$, 117 [$(CH_3)_3CCOS$]$^-$. HPLC t_R 27.3 min. Anal. Calcd for $C_{22}H_{36}N_5O_8PS_2$: C, 44.51; H, 6.11; found: C, 44.48; H, 6.05.

3. Results and discussion

In recent years, considerable efforts have been devoted to improving the therapeutic potential of nucleoside analogues by the use of nucleotide

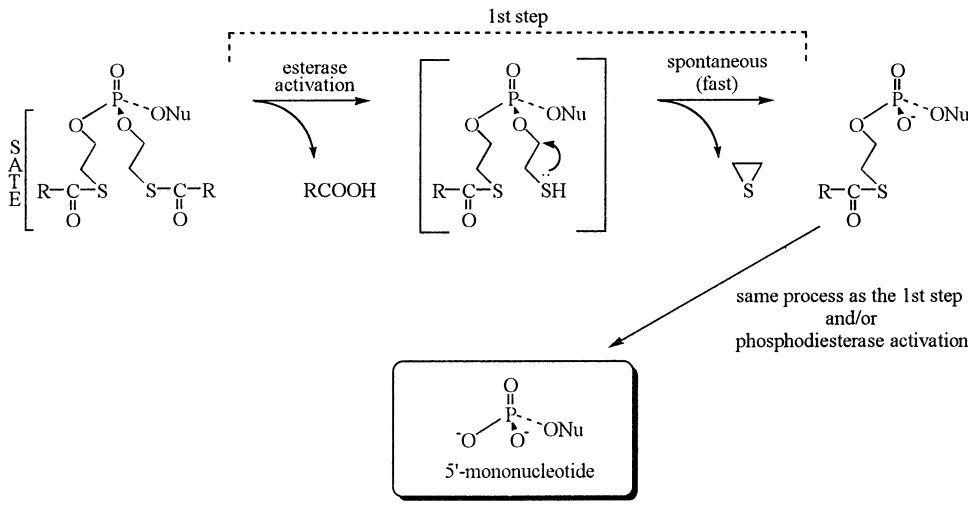

Fig. 2. Proposed mechanisms for the decomposition of a mononucleoside bis(SATE)phosphotriester derivative. Nu, nucleoside analogue.

prodrugs (pronucleotides). Thus, many strategies have been considered to mask or reduce the negative charges of the phosphate function of 5′-mononucleotides (Alexander and Holý, 1994; Krise and Stella, 1996; Périgaud et al., 1996a). Our group has demonstrated that mononucleoside phosphotriesters incorporating a thioethyl chain where the thiol is masked as thioester (SATE groups) were able to liberate the parent 5′-mononucleotide inside the cell (Périgaud et al., 1997). The proposed mechanisms for the decomposition of bis(SATE)phosphotriester derivatives (Fig. 2) involved an esterase-dependent activation process leading to an unstable *O*-2-mercaptoethylphosphotriester, which decomposes spontaneously via intramolecular nucleophilic displacement into the corresponding phosphodiester and ethylene sulfide. Removal of the remaining phosphate protecting group from the resulting phosphodiester derivative following either a similar mechanism and/or phosphodiesterase activity gives rise to the desired parent 5′-mononucleotide. Having shown the validity of a pronucleotide approach using SATE groups as enzyme-labile transient phosphate protections for various anti-HIV 5′-mononucleotides (Gosselin et al., 1996; Périgaud et al., 1996b), we decided to extend the investigation of this approach to the anti-HBV chemotherapy field.

Until recently, where promising results were obtained with some L-nucleosides such as (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (lamivudine, 3TC), attempts to control chronic HBV disease using antiviral chemotherapy have been relatively unsuccessful (Sommadossi, 1994; Shaw and Locarnini, 1995; Alvarez, 1996). Anti-HBV therapy with nucleoside analogues did appear effective in some cases, but always at rather large doses (for a review, see: Rensen et al., 1996). The decrease in serum HBV DNA levels was often transient and treatment of HBV infection was often compromised by associated toxicity. Nevertheless, the discovery that the replication of hepadnaviral genomes involves an obligatory reverse transcription step has stimulated renewed interest in the search of novel compounds with anti-HBV activity and minimal adverse effects on the host upon long-term usage. In common with most of nucleoside analogues, ACV is believed to be biologically active only after phosphorylation to the corresponding 5′-triphosphate, a process which must necessarily occur within the cell because cell membranes are relatively impermeable to polar compounds, including nucleotides. The phosphorylation of nucleoside analogues has been studied extensively in a variety of contexts, and in numerous cases, the initial phosphorylation to the

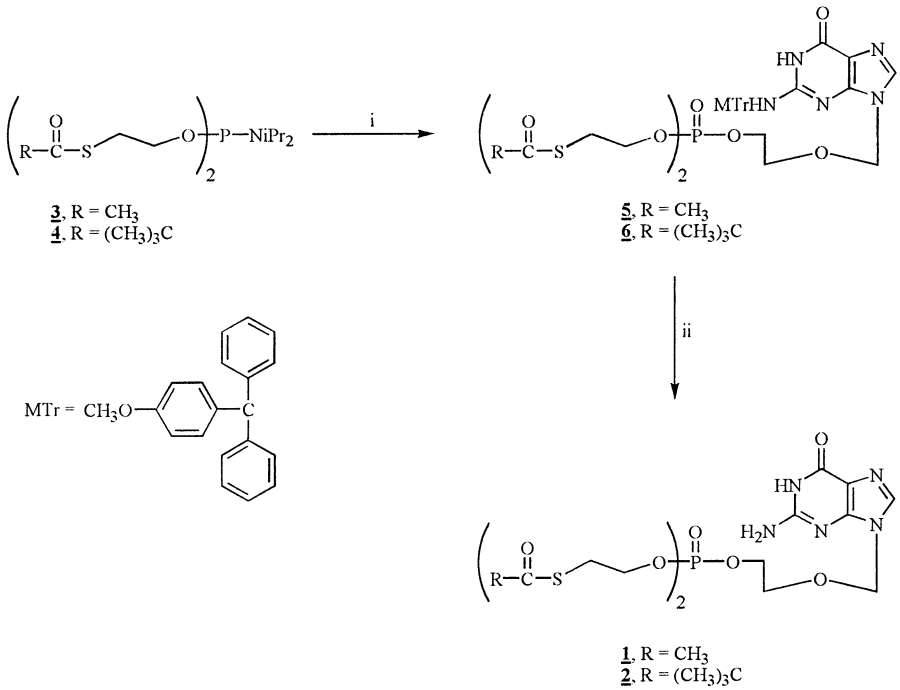

Fig. 3. Synthesis of the bis(SATE)phosphotriester derivatives of ACV. Reaction conditions. (i) N^2-(4-monomethoxytrityl)-9-(2-hydroxyethoxymethyl)guanine, 1*H*-tetrazole/tetrahydrofuran, then $ClC_6H_4CO_3H/CH_2Cl_2$; (ii) CH_3CO_2H/H_2O.

monophosphate level has been identified as the rate-limiting step (Arner and Eriksson, 1995; Shaw and Locarnini, 1995; Johansson and Eriksson, 1996). Studies on the metabolism of ACV in 2.2.15 cells (Shaw et al., 1996) have shown that intracellular concentration of ACVTP, which inhibits efficiently the human HBV DNA polymerase (Hantz et al., 1984), is below the reliable limit of detection. This may be related to the absence of intracellular monophosphorylation of ACV in this cell line, and could explain the negligible efficacy of this nucleoside analogue in patients with chronic hepatitis B. Consequently, the investigation of the SATE biolabile phosphate protection was extended in this study to the 5′-mononucleotide of ACV.

The two bis(SATE)phosphotriester derivatives **1** and **2** were synthesized (Fig. 3) according to a published general procedure (Lefebvre et al., 1995). In order to increase the solubility of ACV in organic solvents and to avoid possible side reactions, the exocyclic guanine amino group was protected by the monomethoxytrityl group. This protecting group could be cleaved by an acidic treatment at the end of the synthesis, after reaction of the appropriate phosphoramidite reagents **3** and **4** with the N^2-protected ACV precursor followed by in situ oxidation.

The pronucleotides **1** and **2** were evaluated for their inhibitory effects on the replication of human HBV in 2.2.15 cells (Table 1) in comparison to ACV, and to 2′,3′-dideoxyguanosine (ddG) as a reference compound (Aoki-Sei et al., 1991; Korba and Gerin, 1992). As expected (Korba and Boyd, 1996), ACV proved to be inactive against HBV-replication in 2.2.15 cells (at concentrations up to 100 μM), whereas the two bis(SATE)-phosphotriester derivatives of ACV emerged as potent inhibitors with 50% effective concentrations (EC_{50}) which were in the same range as the EC_{50} value observed for the reference compound 2′,3′-dideoxyguanosine (ddG). The present results strongly support the hypothesis that the anti-HBV activity of the bis(SATE)-phosphotriester derivatives of ACV may be related, via the intracellular delivery of the 5′-

Table 1
Antiviral activity of the bis(SATE)phosphotriester derivatives of ACV **1** and **2** compared to their nucleoside parent ACV, and to ddG as a reference compound against HBV replication in 2.2.15 cells[a]

Compound	CC_{50} (μM)[b,c]	EC_{50} (μM)[c,d]		EC_{90} (μM)[c,e]		Selectivity index (CC_{50}/EC_{90})[f]	
		Virion[g]	HBV RI[h]	Virion	HBV RI	Virion	HBV RI
1	987 ± 99	0.7 ± 0.1	4.3 ± 0.5	5.1 ± 1.0	15 ± 1.9	194	66
2	1593 ± 131	0.2 ± 0.04	1.1 ± 0.2	7.1 ± 0.8	10 ± 1.1	224	159
ACV	631 ± 40	111 ± 15	>100[i]	>100	>100	na[j]	na
ddG	219 ± 19	1.3 ± 0.2	3.4 ± 0.4	11 ± 1.2	18 ± 2.0	20	12

[a] Confluent cultures of 2.2.15 cells were treated with nine consecutive daily doses of the indicated treatments as previously described (Korba and Gerin, 1992; Korba, 1996). Analysis of HBV DNA was performed 24 h after the ninth day of treatment.
[b] CC_{50}, concentration required to inhibit 50% of cell growth in 2.2.15 cells.
[c] Values presented (± S.D.) were calculated by linear regression analysis using data combined from all treated cultures (six to eight cultures per dilution). Standard deviations were calculated by using the standard error of regression generated from the linear regression analyses.
[d] Concentration required to inhibit 50% of extracellular circular or intracellular replicating HBV DNA.
[e] Concentration required to inhibit 90% of extracellular circular or intracellular replicating HBV DNA.
[f] EC_{90}s were used for the calculation of the selectivity indexes, since at least a 3-fold decrease of HBV RI levels is typically required to achieve statistical significance in this assay system (Korba, 1996).
[g] Extracellular HBV virion DNA.
[h] Intracellular HBV DNA replication intermediates.
[i] >, no significant antiviral effect was observed at 100 μM, the highest concentration tested for antiviral activity.
[j] na, a selectivity index is not available due to a lack of an antiviral effect.

mononucleotide, to an accumulation of the phosphorylated forms of ACV inside the cell. We previously emphasized that when such a SATE pronucleotide approach is applied to a nucleoside analogue which is hampered at its first phosphorylation step, the enhanced antiviral activity could be, in some cases, also associated with a concomitant cytotoxicity owing to the limited selectivity of its 5′-triphosphate derivative against viral polymerase versus human host DNA polymerases (Périgaud et al., 1996c). Despite the fact that ACVTP could interact with cellular DNA polymerases (Johansson, 1993; Ilsley et al., 1995), the cytotoxicity of the pronucleotides **1** and **2** was substantially lower than that of ACV (Table 1). Similar results were noted previously with phospholipid prodrugs of ACV (Hostetler et al., 1993) and of ddG (Korba et al., 1996). Measured in terms of selectivity index, the bis(SATE)-phosphotriesters of ACV **1** and **2** proved to be superior to ddG and emerged as potent and selective inhibitors of HBV replication in vitro. Differences observed between the anti-HBV efficiencies of these two pronucleotides, which differ by a variation of the alkyl chain in the immediate vicinity of the thiol ester functionality, may be explained by their lipophilicity or their decomposition kinetics (in culture medium and inside the cell).

Previous studies in the 2.2.15 cell line have indicated that combinations of nucleosides, especially purine–pyrimidine combinations, can have enhanced activity against HBV replication (Korba, 1996). Treatment of HBV chronic carriers in clinical trials have also given rise to drug-resistant variants (Ling et al., 1996). Combinations of different antiviral agents will probably be necessary to fully control HBV and may help prevent or delay the emergence of drug resistant variants. Among anti-HBV nucleoside analogues, 3TC is the most effective inhibitor of virus replication and it is now undergoing phase III clinical trials (Dienstag et al., 1995). Recently, synergistic inhibition of hepadnaviral replication by 3TC in combination with (9-[2-hydroxy-1-(hydroxymethyl)-ethoxymethyl]guanine (penciclovir, PCV) has

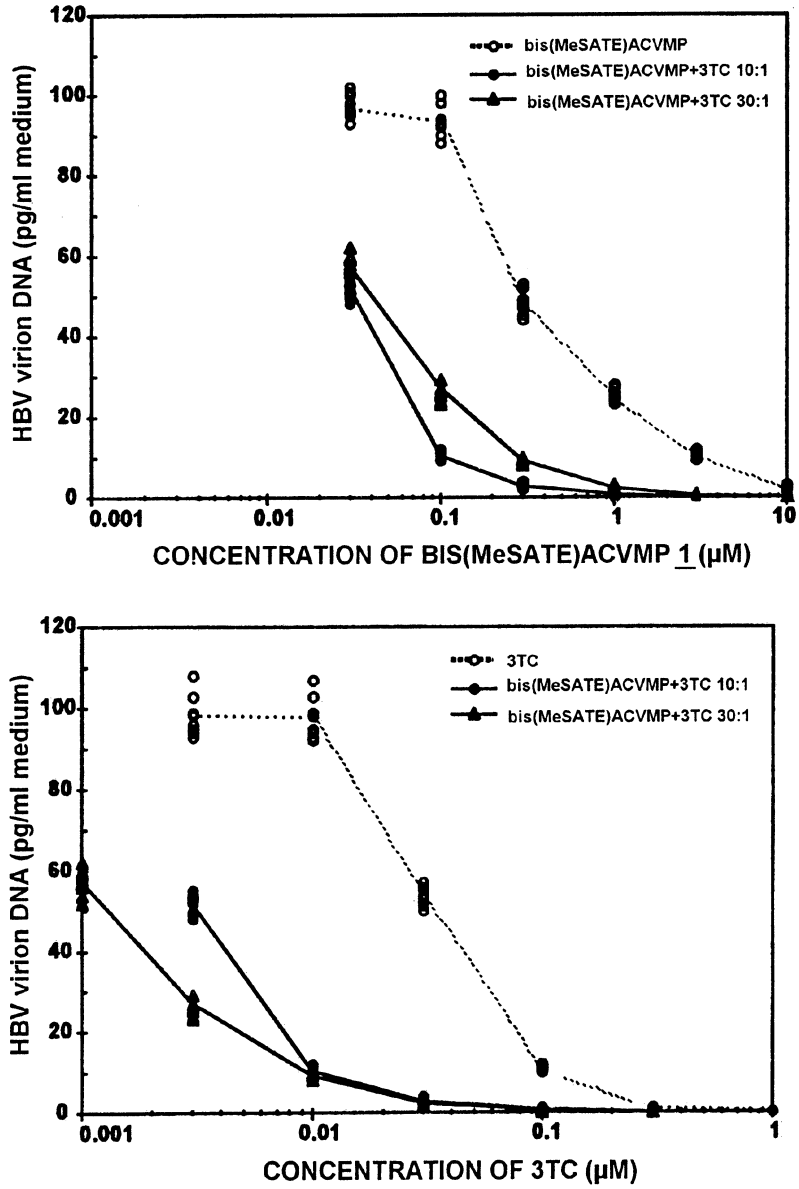

Fig. 4. Effect of combination treatments of bis(MeSATE)ACVMP **1** with 3TC on HBV production by 2.2.15 cells. Cultures were treated as described in Section 2. The levels of HBV virion DNA released into the culture medium by cultures treated with various treatments is shown. Lines denote average values. The two panels display the effect of the combination treatments against the corresponding monotherapies as a function of the concentration of each individual agent.

been reported in vitro (Korba, 1996; Colledge et al., 1997). Like ACV, penciclovir is an acyclic deoxyguanosine analogue originally developed to combat herpesvirus infections (De Clercq, 1997). This result led us to explore the use of bis(MeSATE)ACVMP **1** in combination with 3TC to treat 2.2.15 cells against HBV infection. The combination treatments reduced HBV production in 2.2.15 cells more effectively than the corresponding monotherapies (Fig. 4 and Table 2). Analysis

Table 2
Antiviral activity of bis(MeSATE)phosphotriester derivative of ACV **1** and 3TC, alone and in combination, against HBV replication in 2.2.15 cells[a]

Compound	CC_{50} (μM)[b,c]	EC_{50} (μM)[c,d]	EC_{90} (μM)[c,e]	Selectivity index (CC_{50}/EC_{90})[f]
1	889 ± 77	0.6 ± 0.07	4.0 ± 0.3	222
3TC	1776 ± 149	0.07 ± 0.008	0.2 ± 0.02	8621
1+3TC (10:1)[g]	913 ± 74	0.03 ± 0.004 (0.003)	0.2 ± 0.02 (0.02)	4805
1+3TC (30:1)	861 ± 72	0.07 ± 0.008 (0.002)	0.6 ± 0.05 (0.02)	1484

[a] See Table 1.
[b] See Table 1.
[c] See Table 1.
[d] See Table 1.
[e] See Table 1.
[f] See Table 1.
[g] The molar ratio of **1** to 3TC in each mixture used for the combination treatments is indicated. Values for the combination treatments are expressed as a concentration of **1**. The amounts of 3TC present in the mixtures at the apparent EC_{50} and EC_{90} values for these treatments are listed in parentheses beside the corresponding values for **1** in these mixtures.

of the combination treatments by the CALCUSYN™ software package indicated that 3TC and bis(MeSATE)ACVMP **1** exhibited strong synergistic interactions at all dilutions for both the 10:1 and 30:1 bis(MeSATE)ACVMP:3TC molar ratio mixtures (Fig. 5).

4. Conclusion

The absence of a significant anti-HBV activity in 2.2.15 cells for ACV is consistent with the overall lack of efficacy of ACV against chronic HBV infections in clinical trials. Although some of the numerous clinical studies with this drug reported a modest degree of anti-HBV activity for intravenous treatment with high doses of ACV (which was often associated with unacceptable degrees of nephrotoxicity after prolonged administration), oral administration of ACV had little, if any, effect on HBV replication and is no longer considered to have any clinical benefit for the management of HBV disease in patients with established infections. The absence of activity for ACV, observed during these in vitro and in vivo experiments, was presumably due to its lack of monophosphorylation in HBV-infected cells. Consistent with this hypothesis, the present results report a potent and selective anti-HBV activity of the corresponding bis(SATE)phosphotriesters of ACV in 2.2.15 cells. This anti-HBV activity can be attributed to the unique cellular metabolism of these pronucleotides giving rise to the delivery of ACV 5′-monophosphate inside the infected cells. Finally, data presented here suggest that the use of 3TC and SATE pronucleotides of ACV in combination against HBV infection would be advantageous over 3TC monotherapy. More complete and detailed studies will be necessary to confirm the efficacy of this combination and to determine the mechanism(s) involved in the observed synergy.

In conclusion, the present study demonstrates that the use of a pronucleotide approach extends the antiviral spectrum of a nucleoside analogue. Recently, similar results have been obtained with phospholipid prodrugs of ACV (Hostetler et al., 1997). Given the potency of SATE pronucleotides of ACV against HBV in 2.2.15 cells, further studies including animal experiments are necessary to evaluate the potential of these compounds as anti-HBV agents. Part of these investigations are the subject of the following paper (Hantz et al., 1999). Moreover, the potential of SATE pronucleotides of ACV to restore ACV effectiveness against thymidine kinase-deficient mutants of herpes simplex virus is currently studied.

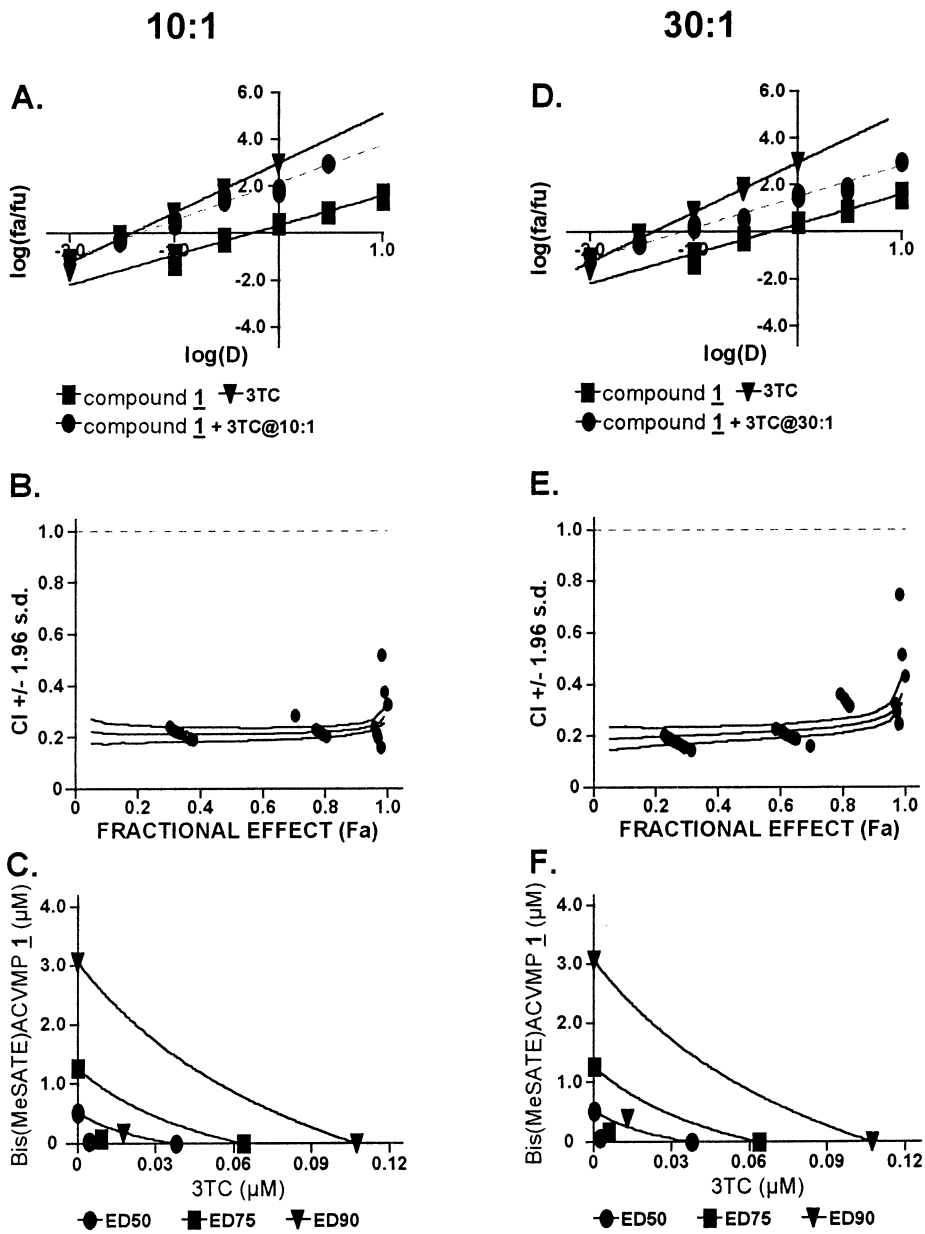

Fig. 5. Analysis of combination treatments with bis(MeSATE)ACVMP **1** and 3TC. Plots were generated by the CALCUSYN™ software package (Biosoft, Cambridge, UK). Panels A–C correspond to the 10:1 molar ratio and panels D–F correspond to the 30:1 molar ratio of the bis(MeSATE)ACVMP:3TC mixtures used for the combination treatments. Analysis of drug interactions in the combination treatments by CALCUSYN™ is based on previously established methods (Chou and Talalay, 1984; Chou, 1991 Belen'kii and Schinazi, 1994). Three separate analyses of the bis(MeSATE)ACVMP **1** and 3TC interactions are displayed: median effect plots (A,D), combination index (CI) plots (B,E), and conservative isobolograms (C, F). For the CI plots, the three lines display median values + 1.96 standard deviation (generated by Monte Carlo statistical analysis): values below 1.0 indicate synergy and values below 0.3 indicate 'strong' interactions (Chou and Talalay, 1984; Chou, 1991; Belen'kii and Schinazi, 1994). For the isobolograms, the ED_{50}, ED_{75} and ED_{90}, values for the combination treatments are shown as individual symbols; values that fall to the lower left of the corresponding lines indicate synergistic interactions (Chou and Talalay, 1984; Chou, 1991).

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