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Inhibitory Effects of Acyclic Nucleoside Phosphonates on Human Hepatitis B Virus and Duck Hepatitis B Virus Infections in Tissue Culture

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The inhibitory effects of the 9-(2-phosphonylmethoxyethyl)adenine-related compounds (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-adenine, (*S*)-9-(3-fluoro-2-phosphonylmethoxypropyl)adenine, (*R*)-9-(2-phosphonylmethoxypropyl)adenine, (*R*)-9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine, and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine on human hepatitis B virus replication in the human hepatoma cell line HepG2 2.2.15 and duck hepatitis B virus infection in primary duck hepatocytes were investigated. (*R*)-9-(2-phosphonylmethoxypropyl-2,6-diaminopurine had the lowest 50% inhibitory concentrations against hepatitis B virus and duck hepatitis B virus, 0.22 and 0.06 μM, respectively, i.e., two- to fivefold lower concentrations than required for (*R*)-9-(2-phosphonylmethoxypropyl)adenine and 9-(2-phosphonylmethoxyethyl)adenine. All compounds were not toxic in vitro at a concentration of 100 μM.

In chronic hepatitis B virus (HBV) infection the basic therapy is the administration of interferon, although complete disappearance of virus markers is seldom observed. Hepatitis B e antigen seroconversion, reflecting a drastic decline of viral replication, is seen in only 20 to 40% of patients with HBV infections.

More potent antiviral drugs are eagerly awaited, and in light of this, the acyclic nucleoside phosphonates described earlier (3, 4) were considered adequate candidates to be further pursued for the treatment of HBV infections.

In an earlier study (6) we investigated the acyclic nucleoside phosphonate 9-(2-phosphonylmethoxyethyl)adenine (PMEA) in two hepatoma cell lines and primary duck hepatocytes. The 50% inhibitory concentrations (IC_{50}) for human HBV and duck HBV (DHBV) were found to be 1.2 and 0.2 μM as measured in HepG2 2.2.15 cells and primary duck hepatocytes, respectively.

Among the acyclic nucleoside phosphonates several other derivatives were found to have high anti-DNA virus activity (herpes group) or antiretrovirus (human immunodeficiency virus [HIV] and Moloney murine sarcoma virus) activity (1). The most effective HIV inhibitors among the acyclic nucleoside phosphonates were evaluated for their anti-HBV activities in the human hepatoma cell line HepG2 2.2.15. Two of the compounds, candidates for application with humans, were also assayed in primary duck hepatocytes infected with DHBV, in anticipation of studies with chronically DHBV-infected ducklings and chronic hepatitis B patients.

PMEA, (*S*)-9-(3-fluoro-2-phosphonylmethoxypropyl)adenine [(*S*)-FPMPA], (*R*)-9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine [(*R*)-PMPDAP], (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA], (*R*)-9-(2-phosphonylmethoxypropyl)adenine [(*R*)-PMPA], and (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine were synthesized by A. Holy, D. Dvorakova, and I. Rosenberg at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic. The structures of the test compounds are depicted in Fig. 1. The HepG2 2.2.15 hepatoblastoma cell line (8) was kindly donated by G. Acs (Mount Sinai Medical Center, New York, N.Y.). Duck hepatocytes were obtained from 2-week-old DHBV DNA-positive ducklings by collagenase perfusion, as described previously (5). The DHBV strain was kindly supplied by K. N. Tsiquaye (London, United Kingdom). Experimental conditions of cell culture for the human hepatoma cell line and the primary duck hepatocytes were as described before (6). The culture medium of HepG2 2.2.15 cells was changed at day 3 after seeding and was supplemented with various concentrations of the drug. The culture medium, including the drug, was changed subsequently every third day. For DHBV-infected duck hepatocytes, the medium was changed at day 1 after seeding and subsequently every other day. From day 3 on, the medium was supplemented with various concentrations of the drug. The 50% cytotoxic concentration was determined by inhibition of 6-[^{3}H]thymidine ([6-^{3}H]dThd) incorporation into HepG2 2.2.15 cell DNA and inhibition of HepG2 2.2.15 cell proliferation as described before (6). For analysis of extracellular HBV DNA and DHBV DNA, cell culture supernatants (HepG2 2.2.15 cells, day 12; duck hepatocytes, day 11), clarified by centrifugation, were prepared for dot blot analysis according to the method of Korba and Milman (7). HBV DNA and DHBV DNA were applied to a Hybond N+ membrane (Amersham Life Science Products) in the Convertible Filtration Manifold System (Bethesda Research Laboratories). Hybridization of HBV DNA sequences was performed with a [^{32}P]dCTP-labelled *Eco*RI fragment of pCP10 containing the full-length 3.2-kb genome of human HBV (2). For hybridization of DHBV DNA sequences, a [^{32}P]dCTP-labelled *Eco*RI fragment of the plasmid pBR322 containing the full-length (3.0-kb) genome of DHBV was used. The plasmid was kindly donated by H. E. Blum (Freiburg, Germany). Labelling reactions were carried out with the Prime-a-Gene labelling system (Promega). For the calculation of the IC_{50} from autoradio-

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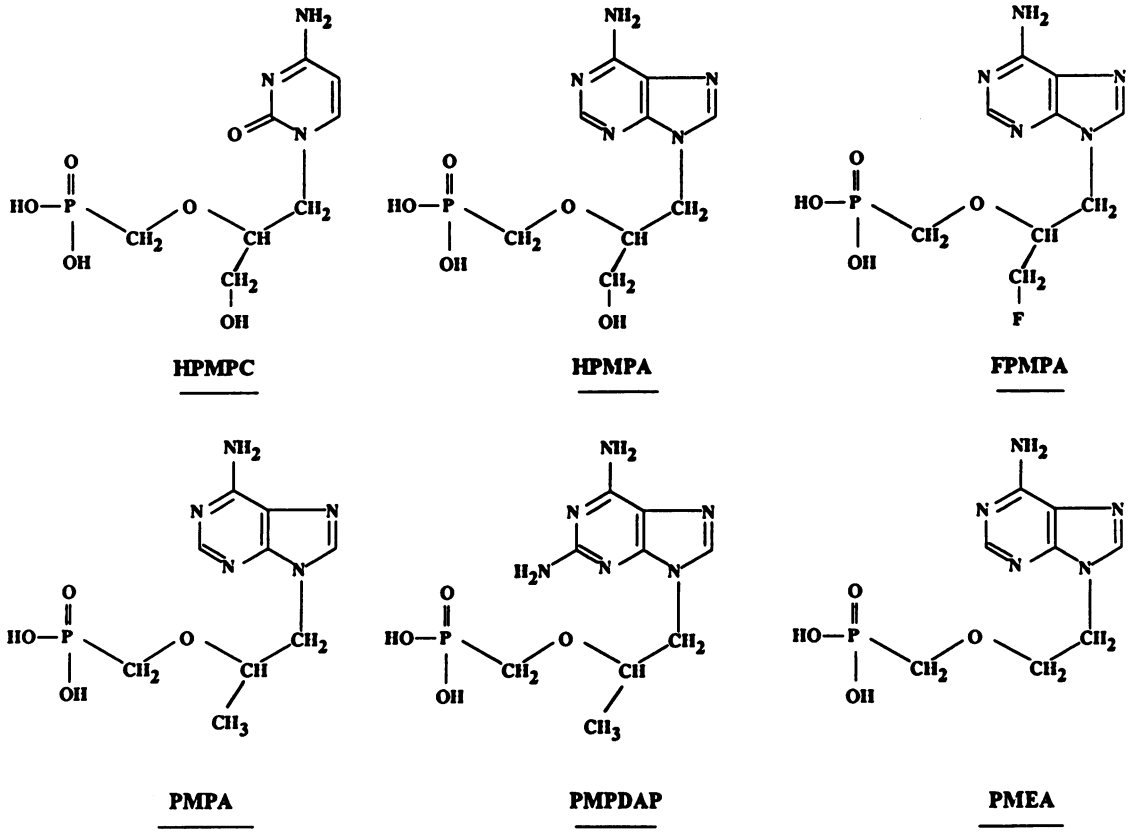

FIG. 1. Structural formulae of the acyclic nucleoside phosphonate derivatives.

grams from dot spot hybridization, an LKB 222-020 Ultra Scan XL laser densitometer was used.

Table 1 presents the results of the evaluation of two 3-hydroxy-2-phosphonylmethoxypropyl and three 2-phosphonylmethoxypropyl derivatives for their inhibitory effects on Dane particle secretion by HepG2 2.2.15 cells. A relatively high IC_{50} was found for (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine, a compound that is currently being tested as a candidate antiviral drug for the treatment of herpesvirus infections. In contrast, (*S*)-HPMPA proved to be highly active against HBV in HepG2 2.2.15 cells and HB611 cells (9) and against DHBV in primary duck hepatocytes (10). Comparable activity was found for PMEA, a compound known to have both DNA virus and retrovirus inhibitory activities (1). (*R*)-PMPA and (*S*)-FPMPA, two effective HIV inhibitors, were also found to have potent inhibitory activities against HBV. None of the compounds evaluated had a marked inhibitory effect on HepG2 2.2.15 cell proliferation at 100 μM (Table 1). No inhibition of [6-^{3}H]dThd incorporation of HepG2 2.2.15 cell DNA was noted at 100 μM.

In the present study, (*R*)-PMPDAP emerged as the most effective inhibitor of HBV in HepG2 2.2.15 cells. (*R*)-PMP DAP has also emerged as the most effective HIV inhibitor among a large series of acyclic nucleoside phosphonates (1).

TABLE 1. Inhibitory effects of acyclic nucleoside phosphonates on extracellular HBV and DHBV production, cell proliferation, and [6-^{3}H]dThd incorporation into DNA

Compound	HBV production, HepG2 2.2.15 cells		DHBV production, primary duck hepatocytes		HepG2 2.2.15 cells	
	IC_{50}	IC_{90}[a]	IC_{50}	IC_{90}	Cell growth, CC_{50}[b]	[6-^{3}H]dThd incorporation into DNA, IC_{50}
PMEA[c]	1.2 ± 0.0	19 ± 13	0.14 ± 0.03	0.36 ± 0.01	>100	>100
(*S*)-HPMPA	1.5				>100	>100
(*S*)-HPMC[d]	14				>100	>100
(*S*)-FPMPA	1.2				>100	>100
(*R*)-PMPA[c]	1.5 ± 0.05	10 ± 2.8	0.11 ± 0.02	0.28 ± 0.02	>100	>100
(*R*)-PMPDAP[c]	0.22 ± 0.03	1.3 ± 0.36	0.06 ± 0.01	0.19 ± 0.01	>100	>100

[a] IC_{90}, 90% inhibitory concentration.
[b] CC_{50}, 50% cytotoxic concentration.
[c] Data are the means from two independent experiments carried out in duplicate.
[d] (*S*)-HPMPC, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine.

Among the compounds [PMEA, (*S*)-HPMPA, (*R*)-PMPA, and (*S*)-FPMPA] that showed comparable IC_{50} (Table 1), PMEA is now in clinical trials for the treatment of HIV infections, (*S*)-HPMPA appears from animal data to be too toxic for clinical use, and (*S*)-FPMPA is rather expensive to produce. Therefore, (*R*)-PMPDAP and (*R*)-PMPA were chosen as the best candidates for further evaluation with duck hepatocytes. As a control, we included PMEA.

In experiments using DHBV-infected duck hepatocytes, IC_{50} of 0.06 and 0.11 μM were found for (*R*)-PMPDAP and (*R*)-PMPA, respectively. For PMEA, an IC_{50} of 0.2 μM was registered earlier (6). The lowest IC_{50} as well as the lowest 90% inhibitory concentration were found once more for (*R*)-PMPDAP. In fact, the same order of antiviral potencies of the test compounds was found in the HBV-infected HepG2 2.2.15 cell and DHBV-infected duck hepatocyte models. These observations may point to the suitability of the DHBV model for determining the relative activities of series of related compounds in vitro.

The 50% cytotoxic concentration of (*R*)-PMPDAP and (*R*)-PMPA was >100 μM for both HepG2 2.2.15 cells and DHBV-infected duck hepatocytes (data not shown).

In summary, the high inhibitory effect of (*R*)-PMPDAP on hepadnavirus infections in both HepG2 2.2.15 cells and DHBV-infected duck hepatocytes is encouraging for further studies with ducks and humans.

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