Inhibitory Effects of Selected Antiviral Compounds on Human Hepatitis B Virus DNA Synthesis

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Hepatitis B virus (HBV) is the causative agent of both the acute and chronic forms of hepatitis. More than 200 million people worldwide are estimated to be chronic carriers of HBV. Efficient anti-HBV drugs have been long awaited. The major problem in the development of such drugs is that an adequate in vitro assay system is not available for the evaluation of potential anti-HBV drugs, because HBV does not readily replicate in culture cells.

Recently, a number of assay systems have been described which seem suitable for evaluation of the inhibitory effects of antiviral compounds against hepadnaviruses, including HBV and duck hepatitis B virus (DHBV) (5, 9, 11, 13, 15). DHBV is able to propagate in primary duck hepatocytes in vitro (12). Selective inhibition of the synthesis of DHBV DNA and core antigen in primary duck hepatocytes can be used as a marker for anti-DHBV activity. Using this assay system, we examined a variety of purine and pyrimidine nucleoside analogs for their inhibitory effects on DHBV replication (15).

Ueda et al. (13) have recently described a human hepatoblastoma cell line (HB611) that contains an integrated HBV genome and that accumulates a significant amount of HBV DNA replicative intermediates. Some of these DNA molecules are packed and released as Dane particles into the cell culture medium. Selective inhibition of the synthesis of these replicative intermediates could serve as a marker for anti-HBV activity. Using the HB611 cell line, we examined the inhibitory effects of selected antiviral compounds on HBV DNA synthesis. The compounds which we evaluated for their inhibitory effect on HBV DNA synthesis were previously examined for their inhibitory effects on DHBV DNA and core antigen synthesis in primary duck hepatocytes (15).

The compounds fell into two categories: phosphorylmethoxyalkylpurines and -pyrimidines and 2',3'-dideoxynucleoside analogs. Their origins were as follows. The phosphorylmethoxyalkylpurines and -pyrimidines [(S)-9-(3-hydroxy-2-phosphonylethoxy)methyl]adenine (S-HPMPA), (S)-1-(3-hydroxy-2-phosphonylethoxy)cytidine [(S)-HPMPC], 9-(2-phosphonomethoxyethyl)-2,6-diaminopurine (PMEDAP), and 9-(phosphonomethoxyethyl)adenine (PMEA) were synthesized by A. Holy and I. Rosenberg at the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, Prague, Czechoslovakia. The 2',3'-dideoxynucleoside analogs 2',3'-dideoxyadenine (DAA), 2',3'-dideoxythymidine (DDT), 2',3'-dideoxy-2',3'-didehydrocytidine (DDC), 2',3'-dideoxy-2',3'-didehydrothymidine (DDT), and 3'-azido-2',3'-dideoxymidine (AZT) were synthesized by P. Herdewijn and A. Van Aerschot at the Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium. 9-β-D-Arabino-furanosyladenine (Ara-A) was obtained from Sigma Chemical Co., St. Louis, Mo.

Test compounds were assayed by a modification of the method described by Ueda et al. (13). The HB611 cell line was generously provided by K. Matsubara, Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan. HB611 cells were seeded in tissue culture trays (diameter, 35 mm) at a density of 5 × 10⁶ cells per well by using Dulbecco modified minimum essential medium supplemented with 10% fetal bovine serum, 100 μg of streptomycin per ml, 100 IU of penicillin G per ml, and 0.2 mg of Geneticin R (GIBCO) per ml. After 2 days of incubation, when the cells were grown to confluence, the medium was withdrawn. Then, the cells were incubated for 12 days, during which time the medium containing the compounds was exchanged every 3 days. The cells were then harvested and total DNA was prepared upon lysis of the cells in 0.5 ml of 10 mM Tris hydrochloride (pH 7.8)–5 mM EDTA–150 mM NaCl–1% sodium dodecyl sulfate–0.1 mg of proteinase K (Sigma) per ml. After phenol-chloroform extraction and ethanol precipitation, the DNA was dissolved in 10 mM Tris hydrochloride (pH 7.8)–1 mM EDTA. Cellular DNA was extracted and digested with the HindIII restriction enzyme (Takara Shuzo). HindIII does not cleave within the HBV genome. An aliquot of the DNA (5 μg) was electrophoresed in a 1.2% agarose gel followed by blotting onto a Hybond N* filter (Amersham Corp., England) by the Southern blot method. The filter was hybridized with a 32P-labeled HBV DNA probe and washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate at 65°C for 30 min. The dried filter was autoradiographed at −70°C by using Kodak CAR-5 film. After autoradiography, each position of bands I, D1, D2, and
The cells were seeded at a rate of $10^5$ cells per well into a 24-well tissue culture tray in 1 ml of growth medium containing various concentrations of the test compounds. After 3 days of incubation at 37°C, the cells were dispersed with 0.5 mg of collagenase per ml and again washed once with growth medium, and the cells were suspended in 2 ml of growth medium. The viable cell number was determined by exclusion of trypan blue (assessed with a 0.1% trypan blue solution) by using a hemacytometer. The minimum cytotoxic concentration or CC$_{50}$ was defined as the concentration required to reduce cell growth by 50%.

A compound that specifically interfered with HBV DNA synthesis may be expected to block the synthesis of the viral replicative intermediates (bands D1, D2, and S) but not the synthesis of the integrated viral DNA (band I) (Fig. 1). As shown in Table 1 and Fig. 2, (S)HPMPA, PMEA, and PMEDAP proved to be highly inhibitory to HBV DNA synthesis in HB611 cells. PMEDAP had the lowest EC$_{50}$ (0.02 μg/ml) and the highest selectivity index (750). Also, (S)-HPMPA and PMEA emerged as potent inhibitors of HBV DNA synthesis (EC$_{50}$, 0.054 to 0.35 μg/ml). The 90% effective concentrations of PMEDAP and PMEA were under 1 μg/ml. HPMPC was 26-fold less inhibitory to HBV DNA synthesis than (S)-HPMPA was.

Of the 2',3'-dideoxynucleoside analogs that were investigated for their inhibitory effects on HBV DNA synthesis, DDC was the most potent (EC$_{50}$, 0.76 μg/ml) and the most selective (Table 1). D4C showed moderate selectivity in inhibiting HBV DNA synthesis, whereas D4T was virtually inactive. Ara-A, an agent that has been used clinically in the treatment of HBV infection, did not show selectivity (selectivity index, <1) as an anti-HBV agent in vitro.

The phosphonylmethoxyalkylpurines and -pyrimidines (i.e., (S)-HPMPA, PMEA, and PMEDAP) are inhibitory to a broad spectrum of DNA viruses, including adenoviruses, poxviruses, iridoviruses (African swine fever virus), and herpesviruses (i.e., herpes simplex virus types 1 and 2, cytomegalovirus, varicella-zoster virus, and Epstein-Barr virus) (1, 4, 6, 14). These compounds inhibited HBV DNA synthesis in HB611 cells at low concentrations (0.02 to 0.35 μg/ml) and had high selectivity indices in vitro. PMEDAP is the most potent and most selective inhibitor of HBV DNA synthesis that has been reported so far. It is noteworthy that, in addition to their anti-HBV activities, PMEA and PMEDAP also exhibited marked inhibitory activities against human immunodeficiency virus (1, 4, 6). Of all the compounds tested, PMEDAP and (S)-HPMPA also proved to be

![FIG. 1. Southern blot analyses of DNA extracted from HB611 cells exposed to PMEDAP and PMEA. Band S corresponds to the single (minus) DNA strand. Band D1 corresponds to partially double-stranded DNA consisting of the minus strand (S strand) and an incomplete plus strand. Band D2 corresponds to the circular double-stranded DNA form, as is normally found in viruses. Band I corresponds to HBV DNA as integrated in the cellular genome.](http://aac.asm.org/)

## TABLE 1. Inhibitory effects of antiviral compounds on HBV DNA synthesis in HB611 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC$_{50}$ (μg/ml)</th>
<th>CC$_{50}$ (μg/ml) by$^a$</th>
<th>Selectivity index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A)$^a$</td>
<td>[6-3H]dThd incorporation (B)</td>
<td>50% HB611 cell growth inhibition (C)</td>
</tr>
<tr>
<td>Ara-A</td>
<td>21.3 ± 6.4</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>DDA</td>
<td>14.2 ± 10.1</td>
<td>200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>DDC</td>
<td>0.76 ± 0.0</td>
<td>320</td>
<td>&gt;200</td>
</tr>
<tr>
<td>D4T</td>
<td>44.7 ± 9.5</td>
<td>200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>D4C</td>
<td>5.6 ± 2.5</td>
<td>200</td>
<td>300</td>
</tr>
<tr>
<td>(S)-HPMPA</td>
<td>0.35 ± 0.44</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>(S)-HPMPC</td>
<td>9.0 ± 1.4</td>
<td>190</td>
<td>60</td>
</tr>
<tr>
<td>PMEA</td>
<td>0.054 ± 0.04</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td>PMEDAP</td>
<td>0.02 ± 0.008</td>
<td>15</td>
<td>35</td>
</tr>
</tbody>
</table>

$^a$ Values are means ± standard deviations of three experiments.

$^b$ The CC$_{50}$ was measured by determining [6-3H]dThd incorporation into host cell DNA (column B) or the inhibition of growth of HB611 cells by 50% (column C).
the most selective in inhibiting the replication of DHBV in primary duck hepatocytes (15). Thus, the antiviral activity spectrum of the phosphononemethoxyalkylpurines not only encompassed adeno-, pox-, irido-, herpes-, and retroviruses but it also extended to the hepadnaviruses.

The mechanism of antiviral action of the phosphononemethoxyalkylpurines and -pyrimidines was not defined. (S)-HPMPA was, as such, taken up by the cells and subsequently converted to its monophosphoryl and diphosphoryl derivatives by cellular enzymes. (S)-HPMPA inhibits the synthesis of herpesvirus DNA at concentrations which are several orders of magnitude lower than the concentration required to inhibit cellular DNA synthesis (1, 6, 14). Thus, (S)-HPMPA discriminates between viral and cellular DNA synthesis, and this may apparently contribute to its selective inhibitory activity against herpesvirus simplex virus, Epstein-Barr virus, and African swine fever virus, as well as other viruses. HPMPA, PMEA, and PMEDAP inhibited not only the D1 and D2 bands but also the S band (Fig. 1). This means that HPMPA, PMEA, and PMEDAP may inhibit both the DNA polymerase and reverse transcriptase activities that are specified by HBV. Further investigations are warranted to determine the interaction of the putative active forms (diphosphoryl derivatives) of HPMPA, PMEA, and PMEDAP with the HBV-associated reverse transcriptase and DNA polymerase.

The 2',3'-dideoxynucleosides (i.e., DDC, D4T, and D4C) are well-known for their selective inhibitory effects on human immunodeficiency virus replication (2, 3, 5, 7, 8, 11, 13). Some 2',3'-dideoxynucleosides, i.e., 2',3'-dideoxyguanosine and 2',3'-dideoxy-2,6-diaminopurineriboside have also reported to inhibit DHBV replication in primary duck hepatocytes (5, 11). Also, Ueda et al. (13) reported that DDC inhibits HBV DNA synthesis at a concentration of 1.6 μM. Our own data have confirmed that DDC is indeed a potent and selective inhibitor of HBV DNA synthesis. DDA was also active, but less so than DDC. D4C and D4T represent two other dideoxynucleoside analogs which are selective inhibitors of human immunodeficiency virus replication (2, 3, 7, 8). Of these two compounds, D4C was effective in inhibiting HBV DNA synthesis, whereas D4T was not.

In conclusion, several antiviral compounds which were previously shown to inhibit the replication of DNA viruses, retroviruses, or both were examined for their inhibitory effects on HBV DNA synthesis in HB611 cells. The most potent and selective inhibitors of HBV DNA synthesis were PMEDAP, PMEA, (S)-HPMPA, DDC, and D4C. These compounds appear to be worth pursuing as candidate drugs for the chemotherapy of HBV infections.

REFERENCES


