In Vitro Activity of 2,4-Diamino-6-[2-(Phosphonomethoxy)Ethoxy]-Pyrimidine against Multidrug-Resistant Hepatitis B Virus Mutants


INSERM, U871, 151 Cours Albert Thomas, 69003 Lyon, France; Université Lyon 1, 69008 Lyon, France; IFR62 Lyon Est, Faculté de Médecine Laennec, 69008 Lyon, France; Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium; Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic; and Hospices Civils de Lyon, Hôtel Dieu Hospital, 69002 Lyon, France

The susceptibilities of drug-resistant hepatitis B virus (HBV) mutants to lamivudine, adefovir, tenofovir, entecavir, and 2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]-pyrimidine (PMEO-DAPym), a novel acyclic pyrimidine analogue, were assessed in vitro. Most drug-resistant mutants, including multidrug-resistant strains, remained sensitive to tenofovir and PMEO-DAPym. Therefore, the latter molecule deserves further evaluation for the treatment of HBV infection.

Treatment of chronic hepatitis B virus (HBV) infection requires the long-term administration of the nucleoside or nucleotide analogs lamivudine [(−)-β-L-2-deoxy-3-thiacytidine], adefovir dipivoxil (9-[2-phosphonomethoxy)ethyl]adenine), entecavir [2-amino-1,9-dihydro-9-[(15,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylene-cyclopentyl]-6H-purin-6-one, monohydrate], or telbivudine (β-L-2′-deoxynucleoside) (28). However, this leads to the emergence of HBV strains harboring mutations within the reverse transcriptase sequence that confer resistance to these drugs (14, 28, 29). The incidence of resistance increases progressively each year, reaching 70% after 4 years of lamivudine therapy and 29% after 5 years of adefovir dipivoxil therapy (9, 14).

Currently, there are two options for the treatment of patients who carry lamivudine-resistant mutants. Lamivudine can be switched to adefovir dipivoxil or entecavir; however, this results in the risk of development of adefovir resistance (7, 8) or entecavir resistance (4, 19) in the long term. Adefovir dipivoxil can also be added to ongoing lamivudine monotherapy (7, 8) to delay the further development of resistance, as both drugs have a favorable cross-resistance profile when they are used in combination (1, 22, 29). However, the emergence of HBV strains simultaneously harboring lamivudine and adefovir resistance mutations was recently reported within the viral quasispecies of a patient who successively failed lamivudine and lamivudine-adefovir dipivoxil add-on therapy (24). The HBV-resistant mutants that are selected after the successive failure of lamivudine and entecavir therapies are resistant to both drugs (20, 23, 26). Thus, the development of novel HBV inhibitors is needed to overcome HBV drug resistance and to design new combination strategies to delay or prevent the development of drug resistance.

Different nucleoside analogs are currently in development. Recently, 2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]-pyrimidine (PMEO-DAPym), an acyclic pyrimidine nucleoside analog phosphonate, was shown to inhibit human immunodeficiency virus (HIV) and HBV replication in vitro with a potency comparable to those of adefovir and tenofovir (R)-9-[2-(phosphonomethoxy)propyl]adenine (2, 11, 27). Tenofovir has been approved as anti-HIV therapy and is in phase III trials for the treatment of HBV infection (25). Moreover, PMEO-DAPym proved to have equipotent activities against wild-type (wt) and lamivudine-resistant rtM204V mutant HBV isolates in inducible transfected hepatoma cell lines (27). In the present study, we investigated in transiently transfected Huh7 cells the cross-resistance profiles of a series of drug-resistant HBV mutants, including multiple-drug-resistant strains, to PMEO-DAPym and to other drugs in parallel assays.

We first determined the effects of the compounds on Huh7 cell viability by determining the concentration of drug that reduced the uptake of neutral red dye by 50% (the 50% cell cytotoxic concentration [CC50]), as described before (10). Transient transfection of Huh7 cells was then performed, as described previously (6), with plasmids containing 1.1 genome unit of a wt or a mutant HBV strain under the control of the chicken beta-actin promoter. One group of constructs contained the genome of HBV laboratory strains (genotype D, serotype ayw), including wt and resistant HBV mutants, obtained by site-directed mutagenesis (lamivudine-resistant mutant rtL180M/M204V, adefovir-resistant mutant rtN236T, and lamivudine- and adefovir-resistant mutant rtL180M/M204V/N236T) (3, 6, 17). The second group of constructs contained HBV genomes cloned from the viral quasispecies of two patients chronically infected with HBV who failed sequential therapy with currently approved HBV inhibitors (23, 24). The following clinical isolates (cloned HBV genomes) were studied: lamivudine-resistant mutants rtL180M/M204V, rtL180M/A181V, and rtV173L/L180M/M204V; lamivudine- and adefovir-resistant mutants rtV173L/L180M/A181V, rtV173L/L180M/A181V/M204V, and entecavir-resistant mutant rtL180M/S202G/M204V. Antiviral assays with transfected cells, purification of intracellular HBV DNA, and analysis of HBV
DNA by Southern blotting were performed as described previously (3, 6).

As shown in Table 1, in Huh7 cells, PMEO-DAPym had little or no effect on cell viability (CC50 >1,000 μM), as was also the case for lamivudine and tenofovir. The CC50 values for entecavir and adefovir were 125 ± 35 μM and 365 ± 120 μM, respectively. Furthermore, PMEO-DAPym had no effect on HBsAg production by wt HBV transfected cells (data not shown). When the anti-HBV activity was assessed, entecavir proved to be the most potent compound, with the lowest 50% effective concentration (EC50), followed by lamivudine, PMEO-DAPym, adefovir, and tenofovir. The EC50 of PMEO-DAPym was three- to fourfold lower than those of adefovir and tenofovir under our in vitro conditions (Table 1). The EC50 of PMEO-DAPym was higher under our in vitro conditions (Table 1). The EC50 of PMEO-DAPym for mutants rtV173L/L180M, rtV173L/L180M/A181V, rtV173L/L180M/A181V/M204V, and rtV173L/L180M/A181V/M204V/N236T were lower than that of tenofovir and similar to that of tenofovir for mutant rtV173L/L180M/A181V/N236T. However, the resistance factor observed for all four lamivudine-adefovir-resistant mutants was slightly higher for PMEO-DAPym compared to that for tenofovir. PMEO-DAPym and tenofovir had greater inhibitory activities than entecavir and similar to that of tenofovir for mutant rtV173L/L180M/A181V/N236T. However, the resistance factor observed for all four lamivudine-adefovir-resistant mutants was slightly higher for PMEO-DAPym compared to that for tenofovir.

PMEODAPym inhibited the replication of both laboratory and clinical lamivudine-resistant HBV variants rtL180M/M204V and rtV173L/L180M/M204V as efficiently as it did with wt HBV (Tables 2 and 3). The rtL180M/A181V mutant displayed a 4.8-fold decreased susceptibility to PMEO-DAPym. However, among the drugs studied, tenofovir was the only one which inhibited this mutant as well as wt HBV (Table 3). Lamivudine-resistant HBV strains show decreased susceptibility to entecavir compared with the susceptibilities of the wt HBV strains (Tables 2 and 3). Interestingly, laboratory HBV strain rtL180M/M204V, which was engineered by site-directed mutagenesis (Table 2), was more susceptible to entecavir than its counterpart derived from one patient (Table 3). Discrepancies between the susceptibilities to entecavir of laboratory- or patient-derived HBV rtL180M/M204V strains have already been observed (20) and may be explained by differences in the genetic backgrounds of the strains outside of the polymerase region that has been cloned.

As reported previously, the rtN236T mutation identified in patients who failed adefovir dipivoxil therapy decreased the sensitivity to adefovir by 3.2- to 7.3-fold (1, 3, 22) and that to tenofovir by 4.5-fold (3) (Table 2). The rtL180M/S202G/M204V mutant, identified in a patient who failed successive lamivudine and entecavir therapies (23), displayed a 210-fold resistance to entecavir and a >100-fold resistance to lamivudine (Table 3). Interestingly, both adefovir- and entecavir-resistant HBV strains were sensitive to PMEO-DAPym (Tables 2 and 3).

All four mutants resistant to lamivudine-adefovir, characterized in a patient who failed sequential therapy, displayed 2.1- to 5.1-fold decreased susceptibilities to PMEO-DAPym, depending on the combination of mutations that they harbored (Table 3). The EC50 of PMEO-DAPym for mutants rtV173L/L180M/A181V, rtV173L/L180M/A181V/M204V, and rtV173L/L180M/A181V/M204V/N236T were lower than that of tenofovir and similar to that of tenofovir for mutant rtV173L/L180M/A181V/N236T. However, the resistance factor observed for all four lamivudine-adefovir-resistant mutants was slightly higher for PMEO-DAPym compared to that for tenofovir. PMEO-DAPym and tenofovir had greater inhibitory activities than lamivudine and entecavir against these multiple-drug-resistant mutants; the factors for resistance to adefovir for these mutants were slightly higher, but the in vivo pharmacological characteristics of adefovir preclude its use at higher dosages (15). The inhibitory activities of the evaluated compounds against the rtV173L/L180M/A181V/N236T mutant (lamivudine and adefovir escape mutant) were in the following order of potency: tenofovir > PMEO-DAPym > entecavir > adefovir > lamivudine.

Our results provide direct information regarding the cross-

### Table 1. Activities of PMEO-DAPym and selected compounds against wild-type HBV replication and cell viability in Huh7 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (μM)</th>
<th>CC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamivudine</td>
<td>1 ± 1.1</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Entecavir</td>
<td>0.3 ± 0.42</td>
<td>125 ± 35</td>
</tr>
<tr>
<td>Adefovir</td>
<td>13 ± 29</td>
<td>365 ± 120</td>
</tr>
<tr>
<td>Tenofovir</td>
<td>16 ± 7.9</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>PMEO-DAPym</td>
<td>4.9 ± 0.6</td>
<td>&gt;1,000</td>
</tr>
</tbody>
</table>

* For each drug, the EC50 value is the mean of the EC50s for wild-type HBV presented in Tables 2 and 3.

### Table 2. Effects of selected anti-HBV drugs on replication of wt HBV and HBV laboratory strains of genotype D carrying lamivudine, adefovir, or lamivudine-adefovir resistance mutations

<table>
<thead>
<tr>
<th>HBV strain</th>
<th>LAM</th>
<th>ADV</th>
<th>TDF</th>
<th>ETV</th>
<th>PMEO-DAPym</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (μM)</td>
<td>FR</td>
<td>EC50 (μM)</td>
<td>FR</td>
<td>EC50 (μM)</td>
</tr>
<tr>
<td>wt</td>
<td>2.48 ± 0.67</td>
<td>1</td>
<td>15.8 ± 1.9</td>
<td>1</td>
<td>10.3 ± 1.3</td>
</tr>
<tr>
<td>ADV</td>
<td>2.65 ± 0.52</td>
<td>1.06</td>
<td>50.3 ± 11</td>
<td>3.2</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>LAM</td>
<td>&gt;100</td>
<td>&gt;40</td>
<td>15.5 ± 1.8</td>
<td>0.98</td>
<td>35.2 ± 5.1</td>
</tr>
<tr>
<td>LAM + ADV</td>
<td>&gt;100</td>
<td>&gt;40</td>
<td>100 ± 20</td>
<td>6.3</td>
<td>45.5 ± 6.1</td>
</tr>
</tbody>
</table>

* LAM, lamivudine; ADV, adefovir; TDF, tenofovir; ETV, entecavir.
  a Lamivudine-resistant (LAM) mutant rtL180M/M204V, adefovir-resistant (ADV) mutant rtN236T, and lamivudine-adefovir-resistant mutant (LAM + ADV) rtL180M/M204V/N236T.
  b Data were reported previously (3).
  c FR, fold resistance, which is equal to (mutant EC50/wt EC50).
  d The values represent the means of at least three independent experiments, each of which was performed in triplicate. For each experiment, the drug-resistant HBV strains and their corresponding wt strain were treated simultaneously with the same range of drug concentrations (from 0 to 100 μM for PMEO-DAPym, lamivudine, adefovir, and tenofovir; from 0 to 10 μM for entecavir), and all the samples were extracted and analyzed by Southern blotting in parallel.
resistance profiles of the lamivudine-, lamivudine-adoefovir-, and entecavir-resistant HBV strains isolated from patients who failed sequential therapy. It is noteworthy that entecavir may not represent the best anti-HBV agent for the treatment of patients who have failed a lamivudine therapy, as lamivudine may lead to the emergence of HBV variants harboring the rL180M/M204V or rL180M/A181V mutation, which impairs the antiviral effect of entecavir (Table 2 and 3). Moreover, long-term entecavir treatment of patients infected with lamivudine-resistant HBV strains leads to the selection of secondary mutations that, in the presence of a genetic background of lamivudine-resistant mutations, confer increased resistance to entecavir (20, 23). Nevertheless, entecavir may be valuable for the treatment of patients who have failed adeovir dipivoxil therapy since mutants harboring the rtN236T mutation, in the absence of the lamivudine resistance mutation rtM204V, retained susceptibility to entecavir (Tables 2 and 3) (3). Tenofovir displayed activity against wt HBV similar to that of adeovir (Table 1) and efficiently inhibited the replication of a series of lamivudine-, adeovir-, lamivudine-adeovir-, and entecavir-resistant HBV strains (Tables 2 and 3). Clinically, tenofovir has been used successfully for the treatment of patients who successively failed lamivudine and lamivudine-adeovir dipivoxil therapy (16, 23, 24). Several clinical reports have suggested the potent anti-HBV activity of tenofovir in patients who failed adeovir therapy and, moreover, the better anti-HBV activity of tenofovir over that of adeovir in patients who failed lamivudine therapy (13, 21), which may be due to the better pharmacokinetic properties of tenofovir. Whether tenofovir may select for drug-resistant mutants in patients remains a matter of controversy (5, 18).

The development of novel strategies for HBV therapy that may be based on the combination of various nucleoside analogs with different cross-resistance profiles will require the discovery of novel HBV inhibitors. We recently demonstrated the in vitro potency of 2',3'-dideoxy-3'-fluoroguanosine for the inhibition of wt, lamivudine-, adeovir-, and lamivudine-adeovir-resistant laboratory HBV strains (12). In the present study, we confirmed the findings of previous studies that showed that PMEO-DAPym is a potent inhibitor of wt HBV in vitro (27). Interestingly, we provide new information showing that PMEO-DAPym inhibits the replication of lamivudine-, entecavir-, adeovir-, and lamivudine-adeovir-resistant mutants almost as efficiently as it does that of wt HBV (Tables 2 and 3). The in vitro cross-resistance profile of PMEO-DAPym for the laboratory and clinical strains studied here proved to be more favorable than the cross-resistance profiles of lamivudine, adeovir, and entecavir and was more or less comparable to that of tenofovir.

Interestingly, PMEO-DAPym efficiently inhibited all HBV variants harboring the rL180M/M204V mutation, which is the most frequently observed lamivudine resistance-conferring mutation found in patients (14, 30) (Tables 2 and 3). Until now, only purine analogs, such as adeovir or tenofovir, have shown activity against the replication of the lamivudine-resistant rL180M/M204V mutant, which is resistant to lamivudine and all known pyrimidine-1-nucleosides (29). Thus, although PMEO-DAPym does not carry a purine base, it exhibits the same cross-resistance profile as purine-based nucleoside phosphonate analogs. This supports our earlier assumption (based on molecular modeling) that the 2,4-diamino-substituted pyrimidine ring of PMEO-DAPym can be viewed as a open-ring analog of the pyrimidine ring system in the 2,6-diaminopurine acyclic nucleoside phosphonate derivatives (27). Although most adeovir- and lamivudine-adeovir-resistant HBV strains retained some degree of susceptibility to adeovir in vitro (Table 2 and 3), the clinical efficacy of adeovir is limited by its nephrotoxicity when the daily dose of adeovir dipivoxil is increased from 10 to 30 mg (15). Under our experimental conditions, tenofovir and PMEO-DAPym exhibited the most favorable in vitro cross-resistance profiles as inhibitors of the replication of multiple-dull-resistant HBV genomes derived from clinical strains.

### Table 3. Effects of selected anti-HBV drugs on replication of HBV mutants derived from the viral quasispecies of chronically infected patients

<table>
<thead>
<tr>
<th>HBV strain</th>
<th>LAM</th>
<th>ADV</th>
<th>TDF</th>
<th>ETV</th>
<th>PMEO-DAPym</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt 1</td>
<td>0.64 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>13.6 ± 4.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>wt 2</td>
<td>0.1 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>10 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>25 ± 7.1</td>
</tr>
<tr>
<td>LAM&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rL180M/M204V</td>
<td>&gt;100&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&gt;1,000</td>
<td>15 ± 6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.5</td>
<td>27 ± 10</td>
</tr>
<tr>
<td>rL180M/A181V</td>
<td>&gt;80 ± 9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>800</td>
<td>27 ± 16&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.7</td>
<td>36 ± 13</td>
</tr>
<tr>
<td>rtV173L/L180M/M204V</td>
<td>&gt;100&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&gt;156</td>
<td>9.8 ± 2.5&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.7</td>
<td>16 ± 5.8</td>
</tr>
<tr>
<td>ETV&lt;sup&gt;v&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rL180M/S202G/M204V</td>
<td>&gt;100&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&gt;156</td>
<td>15 ± 4.5&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.1</td>
<td>27 ± 9.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> LAM, lamivudine; ADV, adeovir; TDF, tenofovir; ETV, entecavir.
<sup>b</sup> LAM<sup>c</sup>, lamivudine resistant; LAM<sup>c</sup> + ADV<sup>v</sup>, lamivudine-adeovir resistant; ETV<sup>v</sup>, entecavir resistant.
<sup>c</sup> FR, fold resistance, which is equal to (mutant EC<sub>50</sub>)/(wt EC<sub>50</sub>). For mutants rtV173L/L180M/M204V and rL180M/S202G/M204V, the corresponding wt strain is wt1 (genotype H) and the fold resistance is equal to (mutant EC<sub>50</sub>)/(wt1 EC<sub>50</sub>). For the other mutants, the corresponding wt strain is wt2 (genotype E) and the fold resistance is equal to (mutant EC<sub>50</sub>)/(wt2 EC<sub>50</sub>).
<sup>d</sup> Values represent the means of at least three independent experiments, each of which was performed in triplicate. For each experiment, the drug-resistant HBV strains and their corresponding wt strains were treated simultaneously with the same range of drug concentrations (from 0 to 100 μM for PMEO-DAPym, lamivudine, adeovir, and tenofovir; from 0 to 10 μM for entecavir), and all the samples were extracted and analyzed by Southern blotting, in parallel.

<sup>e</sup> The data were reported previously (24).
from patients who failed sequential therapy with currently approved HBV inhibitors. Therefore, it will be interesting to determine the pharmacodynamics of PMEO-DAPym in vivo.

In conclusion, the broad inhibitory activity of PMEO-DAPym against HBV drug-resistant mutants and its favorable cytotoxicity profile, observed in tissue culture experiments, warrants the further preclinical evaluation of this compound in animal models of hepadnavirus infections.

This work was part of the activity of the Network of Excellence Virgil, supported by the European Community (ViRgil LSMH-CT-2004-503529) and by the Excellence Prize (HP-AW-CT-2002-9001). This study is part of research project Z440250506 of IOCB Prague, within the framework of the activity of the Center for New Antivirals and Antineoplastics 1M0508 of the Ministry of Education, Youth and Sports. It was also supported in part by the Program of Targeted Projects of the Academy of Sciences of the Czech Republic (IQS 5400550501).

REFERENCES


