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In vitro activity of 2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]-pyrimidine against multidrug-resistant hepatitis B virus (HBV) mutants

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Running title: PMEO-DAPym cross-resistance profile on HBV replication
The susceptibility of drug-resistant hepatitis B virus (HBV) mutants to lamivudine, adefovir, tenofovir, entecavir and 2,4-diamino-6-[2-(phosphonomethoxyethoxy)pyrimidine (PMEO-DAPym), a novel acyclic pyrimidine analogue, was 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 long term administration with nucleos(t)ide analogs [lamivudine [(-)-β-L-2',3'-dideoxy-3’ thiacytidine]), adefovir dipivoxil (9-[2-phosphonomethoxy)ethyl]adenine), entecavir (2-amino-1,9-dihydro-9-[(1S, 3R, 4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate) or telbivudine (β-L-2’-deoxythymidine)] (28). However, this leads to the emergence of HBV strains harbouring mutations within the reverse transcriptase (RT) 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 and 29% after 5 years of adefovir dipivoxil therapy (9, 14). Currently, there are two options to treat patients who carry lamivudine-resistant mutants. Lamivudine can be switched to adefovir-dipivoxil or entecavir with the risk, however, of developing 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 further resistance, as both drugs have a favorable cross-resistance profile when used in combination (1, 22, 29). However, the emergence of HBV strains harbouring simultaneously lamivudine- and adefovir-resistance mutations was recently reported within the viral quasispecies of a patient who successively failed lamivudine and lamivudine plus adefovir dipivoxil add-on therapy (24). The HBV resistant mutants that are selected after successive failure to lamivudine and entecavir 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 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 in vitro human immunodeficiency virus (HIV) and HBV replication with a potency comparable to that of adefovir and tenofovir [(R)-9-[(2-phosphonomethoxy)propyl]adenine] (2, 11, 27). Tenofovir has been approved for HIV
therapy and is in phase III trial for HBV infection (25). Moreover, PMEO-DAPym proved to have equipotent activity against wild-type (wt) and lamivudine-resistant rtM204V mutant HBV 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 this in direct comparison with other drugs in parallel assays.

First, we determined the effect of the compounds on Huh7 cell viability by determining the concentration of drug that reduced the uptake of neutral red dye by 50% (CC$_{50}$), as described before (10). Transient transfection of Huh7 cells was then performed as previously described with plasmids containing 1.1 genome unit of wt or mutant HBV strain under the control of the chicken beta actin promoter (6). 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: rtL180M/M204V; adefovir-resistant: rtN236T; lamivudine+adefovir-resistant: rtL180M/M204V/N236T) (3, 6, 17). The second group of constructs contained HBV genomes cloned from the viral quasispecies of two HBV chronically infected patients 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, rtV173L/L180M/M204V; lamivudine+adefovir-resistant mutants: rtV173L/L180M/A181V, rtV173L/L180M/A181V/M204V, rtV173L/L180M/A181V/M204V/N236T, rtV173L/L180M/A181V/N236T; entecavir-resistant mutant: rtL180M/S202G/M204V. Antiviral assays using transfected cells, purification of intracellular HBV DNA and its analysis by southern blotting were performed as previously described (3, 6).

As shown in Table 1, in Huh7 cells, PMEO-DAPym had little or no effect on cell viability [CC$_{50}$ > 1,000 µM], as was also the case for lamivudine and tenofovir. The CC$_{50}$
value 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 3 to 4-fold lower than that of adefovir and tenofovir, under our in vitro conditions (Table 1). The EC₅₀ of PMEO-DAPym was higher under our in vitro conditions using Huh7 cells by comparison with the results obtained in a stable cell line derived from HepG2 cells (27). This type of EC₅₀ variations between Huh7 and HepG2 cells has already been observed previously with other nucleoside analogs (17); however the ranking of antiviral potency was not affected. This may indicate that the intracellular metabolism including entry, transport, phosphorylation, and pumping out of this nucleoside analogs may depend on the cell lines used for the experiment.

PMEO-DAPym inhibited the replication of both laboratory and clinical lamivudine-resistant HBV variants, rtL180M/M204V and rtV173L/L180M/M204V strains, as efficiently as wt HBV (Tables 2, 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 as compared with wild-type HBV strains (Tables 2, 3). Interestingly, the laboratory HBV strain rtL180M/M204V engineered by site directed mutagenesis (Table 2) is more susceptible to entecavir than its counterpart derived from one patient (Table 3). Discrepancies between the susceptibility to entecavir of laboratory- or patient-derived HBV rtL180M/M204V strains were already observed (20), and may be explained by differences in the genetic background of the strains outside of the polymerase region that has been cloned.
As previously reported, the rtN236T mutation identified in patients who failed adefovir dipivoxil therapy decreased the sensitivity to adefovir by 3.2 to 7.3 (1, 3, 22) and to tenofovir by 4.5-fold (3) (Table 2). The rtL180M/S202G/M204V mutant, identified in a patient who failed successively lamivudine and entecavir therapy (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, 3).

All four lamivudine+adefovir-resistant mutants, characterized in a patient who failed sequential therapy, displayed a 2.1 to 5.1-fold decreased susceptibility to PMEO-DAPym depending on the combination of mutations they harboured (Table 3). The EC$_{50}$ of PMEO-DAPym for mutants rtV173L/L180M/A181V, rtV173L/L180M/A181V/M204V and rtV173L/L180M/A181V/M204V/N236T was lower than that of tenofovir and similar for mutant rtV173L/L180M/A181V/N236T. However, the resistance factor observed for all four lamivudine+adefovir-resistant mutants was slightly higher for PMEO-DAPym as compared to tenofovir. PMEO-DAPym and tenofovir had a greater inhibitory activity on these multiple drug-resistant mutants than lamivudine and entecavir; adefovir had slightly higher resistance factors for these mutants but its in vivo pharmacological characteristics preclude its use at higher dosage (15). The inhibitory activity of the evaluated compounds against the rtV173L/L180M/A181V/N236T mutant (lamivudine and adefovir escape mutant) was ranged in the following order of potency: tenofovir > PMEO-DAPym > entecavir > adefovir > lamivudine.

Our results provide direct information regarding the cross-resistance profile of the lamivudine-, lamivudine+adefovir- and entecavir-resistant HBV strains isolated from patients who failed sequential therapy. Noteworthy, entecavir may not represent the best anti-HBV agent to treat patients who failed a lamivudine therapy, as lamivudine may lead to the emergence of HBV variants harbouring rtL180M/M204V or rtL180M/A181V mutations that...
impair the antiviral effect of entecavir (Tables 2 and 3). Moreover, long-term entecavir
treatment of patients infected with lamivudine resistant HBV strains leads to the selection of
secondary mutations that, on 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 failed adefovir dipivoxil therapy since mutants harbouring the
rtN236T mutation, in absence of the lamivudine-resistant mutation rtM204V, retained
susceptibility to entecavir (Tables 2 and 3) (3). Tenofovir displayed an antiviral activity
against wt HBV similar to adefovir (Table 1), and efficiently inhibited the replication of a
series of lamivudine-, adefovir-, lamivudine+adefovir- 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+adefovir dipivoxil therapy (16, 23, 24).
Several clinical reports suggested a potent anti-HBV activity of tenofovir in patients failing
adefovir therapy and moreover a better anti-HBV activity of tenofovir over adefovir in
patients failing lamivudine therapy (13, 21), which may be due to better pharmacokinetic
properties. 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-resistant profile will require
the discovery of novel HBV inhibitors. We recently demonstrated the in vitro potency of the
2’, 3’-dideoxy-3’-fluoroguanosine to inhibit wt, lamivudine-, adefovir- and lamivudine +
adefovir-resistant laboratory HBV strains (12). In the present study, we confirmed 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-, adefovir- and lamivudine+adefovir-resistant mutants
almost as efficiently as that of wt HBV (Tables 2 and 3). The in vitro cross-resistance profile
of PMEO-DAPym on the laboratory and clinical strains studied here proved to be more favorable than that of lamivudine, adefovir and entecavir, and was more or less comparable to that of tenofovir. Interestingly, PMEO-DAPym efficiently inhibited all HBV variants harbouring the rtL180M/M204V mutations which is the most frequently observed lamivudine-resistant mutant in patients (14, 30) (Tables 2 and 3). Until now, only purine analogs, such as adefovir or tenofovir, have shown activity against the replication of the lamivudine-resistant rtL180M/M204V mutant which is resistant to lamivudine and all known pyrimidine L-nucleosides (29). Thus, PMEO-DAPym, although not carrying a purine base, exhibits the same cross-resistance profile as purine-based nucleoside phosphonate analogs. This supports our earlier assumption that (based on molecular modelling) that the 2,4-diamino-substituted pyrimidine ring of PMEO-DAPym can be viewed as a open-ring analog of the purine system in the 2,6-diaminopurine acyclic nucleoside phosphonate derivatives (27). Although most adefovir- and lamivudine+adefovir-resistant HBV strains retained some degree of susceptibility to adefovir in vitro (Table 2 and 3), its clinical efficacy is limited by its nephrotoxicity when the daily dose of adefovir dipivoxil is increased from 10 to 30 mg (15).

In our experimental conditions, tenofovir and PMEO-DAPym exhibited the most favourable in vitro cross-resistance profiles as inhibitors of the replication of multiple drug-resistant HBV genomes derived from clinical strains 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 further pre-clinical evaluation of this compound in animal models of hepadnavirus infection.
Acknowledgements: This work was part of the activity of the Network of Excellence, “Virgil”, supported by the European Community (ViRgil LSHM-CT-2004-503359), and Descartes Prize (HPAW-CT-2002-9001). This study is part of the research project Z440250506 of IOCB Prague, within the frame 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).
Table 1: Activity of PMEO-DAPym and selected compounds against wild-type HBV replication and cell viability in Huh7 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC$_{50}$ (µM)</th>
<th>CC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamivudine</td>
<td>1 ± 1.1</td>
<td>&gt; 1000</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; 1000</td>
</tr>
<tr>
<td>PMEO-DAPym</td>
<td>4.9 ± 0.6</td>
<td>&gt; 1000</td>
</tr>
</tbody>
</table>

For each drug, the EC$_{50}$ value is the mean of the EC$_{50}$ of wild-type HBV that are shown in Tables 2 and 3.

CC$_{50}$ are means values ± SD for 3 independent experiments performed in quadruplicate.
Table 2: Effect of selected anti-HBV drugs on the replication of wild-type HBV and HBV laboratory strains of genotype D carrying lamivudine (LAM)-, adefovir (ADV)- or lamivudine+adefovir (LAM+ADV)-resistance mutations.

<table>
<thead>
<tr>
<th>HBV strains</th>
<th>LAM&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ADV&lt;sup&gt;b&lt;/sup&gt;</th>
<th>TDF&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ETV&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PMEO-DAPym&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>FR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>FR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
</tr>
<tr>
<td>Wild-type</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-R</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-R</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-R</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>

<sup>a</sup>: FR: Fold resistance = ( mutant EC<sub>50</sub> ) / ( wt EC<sub>50</sub> ).

<sup>b</sup>: Data previously reported in (3).

<sup>c</sup>: Values represent the mean of at least 3 independent experiments, each 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 analysed by southern blotting in parallel.

Lamivudine-resistant (LAM-R) mutant: rtL180M/M204V; Adefovir-resistant (ADV-R) mutant: rtN236T; Lamivudine+adefovir-resistant mutant (LAM+ADV-R): rtL180M/M204V/N236T.
Table 3: Effect of selected anti-HBV drugs on the replication of HBV mutants derived from the viral quasispecies of chronically infected patients.

<table>
<thead>
<tr>
<th>HBV strains</th>
<th>LAM</th>
<th>ADV</th>
<th>TDF</th>
<th>ETV</th>
<th>PMEO-DAPym</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC_{50} (µM)</td>
<td>FR</td>
<td>EC_{50} (µM)</td>
<td>FR</td>
<td>EC_{50} (µM)</td>
</tr>
<tr>
<td>wt 1</td>
<td>0.64±0.17</td>
<td>1</td>
<td>13.6±4.08</td>
<td>1</td>
<td>13.6±4.08</td>
</tr>
<tr>
<td>wt 2</td>
<td>0.1±0.2</td>
<td>1</td>
<td>10±3</td>
<td>1</td>
<td>25±7.1</td>
</tr>
<tr>
<td>LAM-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rtL180M/M204V</td>
<td>&gt;100</td>
<td>&gt;1,000</td>
<td>15±6</td>
<td>1.5</td>
<td>27±10</td>
</tr>
<tr>
<td>rtL180M/A181V</td>
<td>80±9</td>
<td>800</td>
<td>27±16</td>
<td>2.7</td>
<td>36±13</td>
</tr>
<tr>
<td>rtV173L/L180M/M204V</td>
<td>&gt;100</td>
<td>&gt;156</td>
<td>9.8±2.5</td>
<td>0.7</td>
<td>16±5.8</td>
</tr>
<tr>
<td>LAM+ADV-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rtV173L/L180M/A181V</td>
<td>100±5</td>
<td>1,000</td>
<td>48±19</td>
<td>4.8</td>
<td>42±8.1</td>
</tr>
<tr>
<td>rtV173L/L180M/A181V/M204V</td>
<td>&gt;100</td>
<td>&gt;1,000</td>
<td>40±20</td>
<td>4.0</td>
<td>45±13</td>
</tr>
<tr>
<td>rtV173L/L180M/A181V/M204V/N236T</td>
<td>&gt;100</td>
<td>&gt;1,000</td>
<td>77±20</td>
<td>7.7</td>
<td>46±18.3</td>
</tr>
<tr>
<td>rtV173L/L180M/A181V/N236T</td>
<td>&gt;100</td>
<td>&gt;1,000</td>
<td>&gt;100</td>
<td>&gt;10</td>
<td>28±5.6</td>
</tr>
<tr>
<td>ETV-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rtL180M/S202G/M204V</td>
<td>&gt;100</td>
<td>&gt;156</td>
<td>15±4.5</td>
<td>1.1</td>
<td>27±9.8</td>
</tr>
</tbody>
</table>

FR: Fold resistance = (mutant EC_{50})/(wt EC_{50}). For mutants rtV173L/L180M/M204V and rtL180M/S202G/M204V, the corresponding wt strain is wt1 (genotype H) and FR = (mutant EC_{50})/(wt1 EC_{50}). For the other mutants, the corresponding wt strain is wt2 (genotype E) and FR = (mutant EC_{50})/(wt2 EC_{50}).

b: Data previously reported in (24).

c: Values represent the mean of at least 3 independent experiments, each 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 analysed by southern blotting in parallel.

Lamivudine: LAM; Adefovir: ADV; Tenofovir: TDF; Entecavir: ETV; Lamivudine-resistant: LAM-R; Lamivudine+adefovir-resistant: LAM+ADV-R; Entecavir-resistant: ETV-R.
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