Resistance of herpes simplex virus type 1 against different phosphonylethoxyalkyl derivatives of purines and pyrimidines due to specific mutations in the viral DNA polymerase gene

Graciela Andrei, Robert Snoeck, Erik De Clercq, Robert Esnouf, Pierre Fiten and Ghislain Opdenakker

Laboratory of Antiviral Chemotherapy and Laboratory of Molecular Immunology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

Drug-resistant strains of herpes simplex virus type 1 (HSV-1) were selected under the pressure of (S)-3-hydroxy-2-phosphonylethoxypropyl (HPMP) derivatives of cytosine (HPMPC, cidofovir) and adenine (HPMPA) and 2-phosphonylethoxyethyl (PME) derivatives of adenine (PMEA, adefovir) and 2,6-diaminopurine (PMEDAP). HPMPC-resistant (HPMPCr) and HPMPAr strains were cross-resistant to one another, but they remained sensitive to foscarnet (PFA), acyclovir (ACV) and the PME derivatives, while the PMEAr and PMEDAPr mutants were cross-resistant to PFA and ACV. The PMEAr, PMEDAPr and PFAr mutants all revealed a single nucleotide change resulting in a Ser-724 to Asn mutation within the conserved region II of the DNA polymerase. Two HPMPAr clones and one HPMPCr clone possessed single amino acid changes in the DNA polymerase (HPMPAr clone D1, Leu-1007 to Met; HPMPAr clone B5, Ile-1028 to Thr; HPMPCr clone C3, Val-573 to Met). The HPMPCr clone A4 contained two mutations, Ala-136 to Thr and Arg-700 to Met. The mutation at position 136, located outside the catalytic domain of the enzyme, was not detected in other HPMPCr clones, suggesting that this mutation may not be responsible for the resistant phenotype. Residue 573 is located within the 3′ → 5′ exonuclease editing domain close to the catalytically important residues Tyr-577 and Asp-581. Similarly, residue 700 is located in the palm subdomain of the catalytic domain, adjacent to the Asp residues 717, 886 and 888 that are vital for polymerase activity. The HPMPAr mutations at residues 1007 and 1028, beyond the last conserved region, still fall within the thumb subdomain of the catalytic domain. The different drug-resistant mutants varied in neurovirulent behaviour, the HPMPCr strains showing reduced neurovirulence compared with the wild-type.

Acyclovir (ACV) has been shown to be effective in the prophylaxis and treatment of mucocutaneous HSV infections in immunocompromised patients (Whitley & Gnann, 1992). The development of resistance to ACV has been most extensively studied (Coen, 1996a; Collins & Darby, 1991). ACV is phosphorylated to its monophosphate form by the virus-encoded thymidine kinase (TK) and, after further phosphorylation to the triphosphate form by cellular enzymes, it inhibits herpesvirus DNA polymerases specifically by competing with the binding of natural triphosphates and their subsequent insertion into growing DNA strands. Three different mechanisms are recognized that render HSV resistant

Introduction

Herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) lead to diseases that are usually self-limiting in the normal population, but they can be major causes of morbidity and mortality in the immunocompromised host. Prolonged antiviral treatment is often required for the clinical management of herpesvirus infections in these patients, and this favours the emergence of drug-resistant strains.

Author for correspondence: Graciela Andrei.
Fax +32 16 337340, e-mail graciela.andrei@rega.kuleuven.ac.be
to ACV. Most of the acyclovir-resistant (ACV\textsuperscript{R}) strains that have been isolated, either from cell culture or from patients, lack a functional TK (TK\textsuperscript{-} mutant) and are resistant because of the inability to monophosphorylate ACV (McLaren et al., 1985; Chatis & Crumpacker, 1991; Hill et al., 1991). A few isolates have been found to have a TK with altered substrate specificity, resistant viruses producing a TK enzyme that is functional but that lacks the ability to phosphorylate ACV (Ellis et al., 1987; Nugier et al., 1991). Finally, resistance to ACV due to mutations that alter binding and utilization of ACV by the viral DNA polymerase has been described (Parker et al., 1987; Collins et al., 1989; Sacks et al., 1989).

Foscarnet (PFA) is an alternative therapeutic modality for the treatment of TK\textsuperscript{-}, ACV\textsuperscript{R} HSV infections, since it does not require activation by the viral TK (Safrin et al., 1991). Unfortunately, administration of PFA often causes renal failure and alterations in the plasma calcium and phosphorus levels. In addition, resistance to both ACV and PFA has been described in the clinic (Safrin et al., 1994; Safrin, 1996). A new approach to the therapy of TK\textsuperscript{+} as well as ACV\textsuperscript{R} and ACV\textsuperscript{R}/PFA\textsuperscript{R} mucocutaneous HSV infections is based on the use of cidofovir [HPMPC; (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine], which does not depend on the viral TK for its activity (Hitchcock et al., 1996; Nugier et al., 1991). HPMPC has proven to be effective in the treatment of progressive mucocutaneous infections due to ACV\textsuperscript{R} and ACV\textsuperscript{R}/PFA\textsuperscript{R} HSV in immunocompromised patients (Snoeck et al., 1993; Lalezari et al., 1997). It is one of the leading compounds of a new series of antiviral molecules, the acyclic nucleoside phosphonate (ANP) analogues. Within this class of compounds, (S)-3-hydroxy-2-phosphonylmethoxypropyl (HPMP) and 2-phosphonylmethoxyethyl (PME) derivatives of both purines and pyrimidines have been synthesized (De Clercq et al., 1986, 1987). The HPMP derivatives have potent and selective activity against a broad spectrum of DNA viruses, including herpes-, hepapna-, irido-, pox-, adeno-, papilloma- and polyomaviruses (Hitchcock et al., 1996; Naesens et al., 1997). HPMPC has been approved for the treatment of cytomegalovirus retinitis in AIDS patients (Safrin et al., 1997) and is being evaluated for the treatment of other herpesviruses including HSV, varicella-zoster virus and Epstein–Barr virus, as well as adeno-, papilloma- and polyomaviruses. In contrast to the HPMP derivatives, the PME derivatives show marked and selective activity against retroviruses. Like the HPMP derivatives, the PME derivatives are also active against herpes-, hepapna- and iridoviruses, but unlike the HPMP derivatives they do not inhibit adeno- or poxviruses. PME-adeneine (PMEA), as its oral prodrug form (adefovir dipivoxil), has proceeded to phase III clinical trials in both the USA and Europe for the treatment of human immunodeficiency virus.

Phosphonylmethoxalkyl derivatives of purines and pyrimidines, which can be considered as analogous to the monophosphate forms of acyclic nucleosides, are further phosphorylated to their active metabolites by cellular enzymes. Thus, they circumvent the need for activation by the virus-specific TK and hence retain their activity against TK-deficient or -altered strains of HSV (Hitchcock et al., 1996; Naesens et al., 1997). Furthermore, it appears that HSV mutants with deficient or altered TK activity are more susceptible to HPMPC, due to a reduction in the dCTP pools, which normally depend on TK (Mendel et al., 1995). The antiviral effect of the ANP analogues is the result of selective inhibition of the viral DNA polymerase by their diphosphate metabolites. Based on the structural resemblance to natural deoxynucleoside triphosphates, the diphosphate metabolites act both as competitive inhibitors and as alternative substrates during the DNA polymerase reaction (Foster et al., 1991; Xiong et al., 1996, 1997). These diphosphate forms inhibit HSV DNA polymerase at concentrations that are 50- to 600-fold lower than those needed to inhibit human α, β and γ DNA polymerases (Merta et al., 1990; Ho et al., 1992).

The isolation and characterization of drug-resistant mutants of HSV DNA polymerase can be useful in elucidating the mechanism(s) of selective drug action, in assessing the potential for drug resistance in the clinic and its possible avoidance and in evaluating the structure–function relationship of the polymerase gene domains (Coen, 1996a).

The aim of the present study was to determine the molecular basis of HSV-1 resistance to both HPMP and PME derivatives by the identification of the nucleotide changes that occurred in the DNA polymerase genes of HSV-1 mutants selected under drug pressure in vitro. Also, the neurovirulence of the different mutants was evaluated in a mouse model. The relationship between cross-resistance for the different drug classes in vitro and neurovirulence in vivo may form a biochemical basis for combination chemotherapy with far-reaching implications in clinical practice.

**Methods**

- **Cells and viruses.** Human embryonic lung (HEL) fibroblasts and Vero cells were maintained in minimum essential medium supplemented with 10% inactivated foetal calf serum, 1% l-glutamine and 0.3% sodium bicarbonate. The HSV-1 laboratory strain KOS was used.

- **Compounds.** The sources of the compounds were as follows: acyclovir [ACV, 9-(2-hydroxyethoxymethyl)guanine], Wellcome Research Laboratories, Research Triangle Park, NC, USA; foscarnet [phosphonoformate sodium salt, PFA] and phosphonoacetic acid (PAA), Sigma; PMEA [9-(2-phosphonylmethoxyethyl)adenine] and HPMPC [(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine], Gilead Sciences, Foster City, CA, USA; HPMPC [(S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine] and PMEDAP [9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine], A. Holy, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic.

- **Selection of drug-resistant strains.** The drug-resistant virus strains were obtained by serial passage of the reference HSV-1 KOS strain in the presence of increasing concentrations of the compounds. The virus was passaged in Vero cells in the presence of the compounds, starting at the IC\textsubscript{50}. The cell cultures were incubated until virus CPE was
maximal, and the drug concentration was increased 2-fold with every subsequent passage of the virus. After reaching the highest possible concentration for each compound (100 µg/ml for PMEA and PMEDAP, 200 µg/ml for PFA and 40 µg/ml for HPMPC and HPMPA), a last passage was done in drug-free medium in order to obtain the virus stock. The various drug-resistant HSV-1 strains, denoted PFAr, PMEA, PMEDAPr, HPMPCr and HPMPAr, were titrated and subsequently tested for their sensitivity in vitro to a broad range of antiviral compounds. Drug resistance was defined taking into consideration the potency of each anti-HSV compound. Thus, drug resistance corresponded to an increase in IC50 of at least 10-fold for the HPMP derivatives, at least 5-fold for the PME derivatives and at least 3-fold for the pyrophosphate analogues. In the case of ACV, a 5- to 10-fold increase in IC50 was regarded as indicative of resistance due to alterations at the DNA polymerase level.

- **Plaque purification.** Each drug-resistant strain was plaque-purified by standard procedures. Several individual PFA, PMEA, PMEDAP, HPMPC and HPMPA clones were collected, amplified and tested for their sensitivity in vitro to various antiviral compounds.

- **Antiviral assays.** Drug sensitivity of the different drug-resistant HSV strains was determined by virus CPE reduction assays in HEL cells. Confluent HEL cells were inoculated with the different virus strains at an input of 100 CCID50 (1 CCID50 corresponds to the virus dose infective for 50% of the cell cultures). The IC50 was defined as the concentration required to reduce virus CPE by 50%. The IC50 values for the individual replicates represent the means from at least three independent experiments.

- **Cloning and sequencing of the DNA polymerase gene variants.** HSV DNA was prepared directly from virus produced after infecting Vero cell cultures with the KOS strain or the different mutants. Two HPMPC clones (clone A4 and clone C3), two HPMPA clones (clone B5 and clone D1), one PFA clone (clone A), one PMEA clone (clone B) and one PMEDAP clone (clone C) were selected for cloning and sequencing of the DNA polymerase genes. To avoid the introduction of mutations by PCR amplification, all cloning experiments were executed directly on viral DNA. Viral DNA from the different strains was digested with BamHI and fragments were separated by electrophoresis in agarose gels. A 3-4 kb BamHI fragment that contains about 87% of the HSV-1 DNA polymerase gene coding region was purified and further digested with SstI. The two resulting fragments of 2-0 and 1-4 kb from each virus were purified and ligated to pUC18 cleaved with BamHI and SstI. The ligation mixtures were used to transform *Escherichia coli* (strain DH5α) made competent by the method of Hanahan (1985). Ampicillin-resistant colonies were identified and screened for plasmids containing the appropriate fragments of 2-0 and 1-4 kb. Plasmid DNA was prepared and aliquots of either 6 µg (for T7 DNA polymerase sequencing) or 500 µg (for cycle sequencing) were used. The total insert of the plasmid DNAs was sequenced by the dideoxynucleotide chain termination method starting with fluorescent universal forward and reverse M13 primers, followed by primer walking with internal fluorescent primers (AutoRead sequencing kit, Pharmacia Biotech) designed according to the wild-type HSV-1 strain KOS sequence. The sequencing reaction products were run on an automated laser fluorescent DNA sequencer under standard conditions (Chen & Seeberg, 1985).

- **Screening of identified mutations in various plaque-purified virus clones.** To determine the presence of specific mutations in various plaque-purified drug-resistant HSV-1 isolates, cycle sequencing of total viral DNA was performed (Thermo sequencing fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP, Pharmacia Biotech). We used specific primers that allowed the determination of the nucleotide sequence of the region of the viral DNA polymerase in which the mutations had occurred.

- **Determination of the in vivo pathogenicity of the plaque-purified drug-resistant strains.** Virus stocks of the plaque-purified drug-resistant strains were titrated in HEL cells by plaque formation and the virus titre was expressed in p.f.u./ml. In parallel, adult NMRI mice were inoculated intracerebrally with 10-fold dilutions of each virus stock. Ten mice were used per dilution. Mortality was recorded over a period of 20 days and virus titre was expressed in LD50/ml. The pathogenicity index for the different strains was calculated as the log (p.f.u./LD50).

**Results**

**Phenotypic and genotypic characterization of PMEA, PMEDAP and PFA HSV-1 clones**

The plaque-purified PMEA, PMEDAP and PFA clones showed cross-resistance to PMEA, PMEDAP, the pyrophosphate analogues PFA and PAA and ACV. They remained sensitive to the HPMP derivatives, although an increase in the IC50, varying from 2- to 8-fold, was seen with HPMPC. It should be noted that the increase in the IC50 values for HPMPA was not higher than 2-5-fold (Tables 1 and 2).

The DNA polymerase genes of one PMEA clone (clone B), one PMEDAP clone (clone C) and one PFA clone (clone A) were cloned and sequenced. Sequence analysis of the DNA polymerase genes of these mutants revealed a single nucleotide change that resulted in a Ser-724 to Asn mutation within the DNA polymerase level.

Drug resistance was defined taking into consideration the potency of each anti-HSV compound. Thus, drug resistance corresponded to an increase in IC50 of at least 10-fold for the HPMP derivatives, at least 5-fold for the PME derivatives and at least 3-fold for the pyrophosphate analogues. In the case of ACV, a 5- to 10-fold increase in IC50 was regarded as indicative of resistance due to alterations at the DNA polymerase level.

**Phenotypic and genotypic characterization of HPMPC and HPMPA HSV-1 clones**

The HPMPC and HPMPA clones proved to be resistant only to HPMPC and HPMPA, remaining sensitive to ACV, the PME derivatives and the pyrophosphate analogues PFA and PAA (Tables 1 and 2). Indeed, some of the HPMPC clones appeared to be hyperresistant to the pyrophosphate analogues.

Two HPMPC virus isolates (clone A4 and clone C3) and two HPMPA isolates (clone B5 and clone D1) were selected for molecular cloning and sequencing of the DNA polymerase genes. As shown in Table 1, the HPMPA clones B5 and D1 and the HPMPC clone C3 possessed single amino acid changes in the DNA polymerase. The HPMPA clones B5 and D1 showed an Ile-1028 to Thr change and a Leu-1007 to Met change, respectively, while the HPMPC clone C3 presented a Val-573 to Met mutation. The HPMPC clone A4 contained two mutations, Ala-136 to Thr and Arg-700 to Met.

Several HPMPC and HPMPA mutant viruses were selected for the determination of their drug-susceptibility
Table 1. Mutations in the DNA polymerases of various drug-resistant HSV-1 clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>PFA</th>
<th>PMEDAP</th>
<th>PAA</th>
<th>HPMPA</th>
<th>HPMPC</th>
<th>PMEA</th>
<th>IC₅₀ (µg/ml) mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>E3</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>A4</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>A3</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>A2</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>A1</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>KOS</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>25 ± 4</td>
</tr>
</tbody>
</table>

Locations of the mutations in the HSV-1 DNA polymerase that confer resistance to acyclic nucleoside analogues

The Ser-724 to Asn mutation, which confers resistance to ACV in addition to PMEA, PMEDAP and PFA, is mapped to conserved region II of the HSV-1 DNA polymerase (Fig. 1). The serine at position 724 is highly conserved in all herpesviral DNA polymerases and in almost every other polymerase that shares this region of sequence similarity.

The mutations found in the HPMPC⁺ and HPMPA⁺ clones are mapped to non-conserved regions of the DNA polymerase or to non-conserved positions within conserved regions. Thus, the Arg-700 to Met mutation found in the HPMPC⁺ clone A4 is mapped to conserved region II, but in a position that is highly variable among the cellular and viral polymerases. The HPMPC⁺ clone A4 also presented an Ala-136 to Thr change, which is not located in the proposed catalytic domain of the DNA polymerase. This amino acid change was not confirmed in other HPMPC⁺ isolates that showed the Arg-700 to Met change, suggesting that the Ala-136 to Thr mutation may not be responsible for the resistant phenotype.

The mutations found in the HPMPC⁺ clone C3 and the HPMPA⁺ clones B5 and D1 (at positions 573, 1028 and 1007, respectively) are located in non-conserved regions within the DNA polymerase. Position 573 is located just before two well-conserved residues, Tyr-577 and Asp-581, which are within conserved region A (Fig. 1). HSV-1 DNA polymerase has a C-terminal UL42-binding domain. Positions 1007 and 1028 lie beyond the last region of conserved sequence (region V) and before the UL42-binding domain.

Neurovirulence

Neurovirulence of the different drug-resistant HSV-1 mutants was evaluated by intracerebral inoculation of the
Table 2. Phenotypic and genotypic characterization of various PFA\(^{-}\), PMEA\(^{-}\) and PMEDAP\(^{-}\) HSV-1 clones

The presence of the Ser to Asn change at position 724 was determined by direct cycle sequencing of total viral DNA.

<table>
<thead>
<tr>
<th>Clone</th>
<th>ACV</th>
<th>PFA</th>
<th>PAA</th>
<th>PMEA</th>
<th>PMEDAP</th>
<th>HPMPPC</th>
<th>HPMPA</th>
<th>Ser-724 to Asn</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFA(^{-}) clone G</td>
<td>0.14 ± 0.08</td>
<td>93 ± 10</td>
<td>41 ± 13</td>
<td>42.5 ± 10.6</td>
<td>18 ± 3</td>
<td>0.30 ± 0.02</td>
<td>0.045 ± 0.007</td>
<td>+</td>
</tr>
<tr>
<td>PFA(^{-}) clone H</td>
<td>0.11 ± 0.01</td>
<td>105 ± 21</td>
<td>47 ± 5</td>
<td>47 ± 4</td>
<td>20 ± 10</td>
<td>0.41 ± 0.13</td>
<td>0.006 ± 0.05</td>
<td>+</td>
</tr>
<tr>
<td>PMEA(^{-}) clone A</td>
<td>0.05 ± 0</td>
<td>98.5 ± 51.6</td>
<td>50 ± 0</td>
<td>57 ± 10</td>
<td>18 ± 3</td>
<td>0.38 ± 0.04</td>
<td>0.05 ± 0</td>
<td>+</td>
</tr>
<tr>
<td>PMEA(^{-}) clone D</td>
<td>0.08 ± 0.04</td>
<td>84 ± 16</td>
<td>26 ± 7</td>
<td>51 ± 22</td>
<td>21 ± 5</td>
<td>0.44 ± 0.09</td>
<td>0.081 ± 0.062</td>
<td>+</td>
</tr>
<tr>
<td>PMEA(^{-}) clone G</td>
<td>0.08 ± 0.04</td>
<td>76 ± 25</td>
<td>32 ± 8</td>
<td>51 ± 9</td>
<td>20 ± 9</td>
<td>0.15 ± 0.07</td>
<td>0.035 ± 0.02</td>
<td>+</td>
</tr>
<tr>
<td>PMEDAP(^{-}) clone A</td>
<td>0.48 ± 0.28</td>
<td>120 ± 57</td>
<td>41 ± 9</td>
<td>145 ± 65</td>
<td>120 ± 53</td>
<td>0.27 ± 0.14</td>
<td>0.09 ± 0.04</td>
<td>+</td>
</tr>
<tr>
<td>PMEDAP(^{-}) clone B</td>
<td>0.18 ± 0.10</td>
<td>81 ± 36</td>
<td>42 ± 10</td>
<td>40 ± 7</td>
<td>26 ± 8</td>
<td>0.62 ± 0.14</td>
<td>0.09 ± 0.02</td>
<td>+</td>
</tr>
<tr>
<td>PMEDAP(^{-}) clone G</td>
<td>0.76 ± 0.48</td>
<td>113 ± 35</td>
<td>41 ± 7</td>
<td>163 ± 52</td>
<td>&gt; 50 ± 0</td>
<td>0.40 ± 0.04</td>
<td>0.072 ± 0.04</td>
<td>+</td>
</tr>
</tbody>
</table>

viruses into mice. The LD\(_{50}\) values for the different isolates were determined, and the ratio p.f.u./LD\(_{50}\) was monitored as a parameter of neurovirulence. A TK\(^{-}\) ACV\(^{+}\) KOS mutant with a reduced neurovirulence (increase in the ratio of p.f.u. to LD\(_{50}\)) was included as control (Fig. 2). The strains resistant to PFA, PMEA and PMEDAP showed high neurovirulence similar to, or slightly lower than, that of the parental neurovirulent KOS strain. In contrast, the HPMP\(^{+}\) strains (clone C3, clone A4 and clone D) and HPMPA\(^{-}\) strains (clone A3 and clone B5) showed a reduction in neurovirulence (ratio of p.f.u. to LD\(_{50}\) > 1-9). Two of the HPMPA\(^{-}\) clones (clone D1 and clone B5) showed high neurovirulence similar to that of the KOS strain.

Discussion

We have observed that HPMP\(^{+}\) and HPMPA\(^{-}\) strains of HSV-1 selected in vitro are cross-resistant to one another, but still sensitive to PFA, ACV and the PME derivatives, while the PMEA\(^{-}\) and PMEDAP\(^{-}\) strains are cross-resistant with the pyrophosphate analogues PFA and PAA. The pattern of drug susceptibility profiles reported here with plaque-purified viruses is in agreement with those that we have described for the original mutant viruses (Andrei et al., 1995). The lack of cross-resistance between the HPMP derivatives and the PME derivatives indicates that these subclasses of ANP analogues differ in their mode of interaction with the viral DNA polymerase. Therefore, sequencing of the viral DNA polymerase gene to identify drug-resistance mutations was useful for identifying amino acids that are involved directly or indirectly in recognition of the different subclasses of ANP analogues.

HSV-1 DNA polymerase is a 1235 amino acid multifunctional enzyme with a 5’ → 3’ exonuclease/RNase H function, a 3’ → 5’ exonuclease editing function, a deoxyribonucleotide polymerizing (catalytic) function and a UL42-binding domain (Earl et al., 1986; Larder et al., 1987; Coen, 1996b). This polymerase belongs to a large family of polymerases with seven regions having somewhat conserved nucleotide sequences. The regions are numbered I to VII on the basis of degree of conservation among the DNA polymerase genes, with region I being the most conserved. The recent report of the crystal structure of one member of this family of polymerases, that of bacteriophage RB69 (Wang et al., 1997), allows these conserved regions to be correlated with structural and functional roles. Regions I and II are parts of the polymerase palm subdomain and contain the three Asp residues (717, 886 and 888 in HSV-1) essential for polymerase activity. Regions III and VI are located at the base of the fingers subdomain and regions V and VII are at the base of the thumb subdomain. These four regions appear to flank the catalytic site in the palm subdomain and may play a role in positioning the template and primer strands. Region IV is part of the 3’ → 5’ exonuclease editing domain and these residues may play a more structural role. Finally, a further region, A, has been identified. Region A is now recognized as being part of a larger region called δ-region C that is shared by polymerases related to eukaryotic DNA polymerase δ (Coen, 1996b). Although there is only low sequence conservation, this region appears to be part of the 3’ → 5’ exonuclease editing domain that includes the conserved catalytic residues Tyr-577 and Asp-581 (Fig. 1).

Most work concerning mutations in the HSV DNA polymerase has been done with ACV and PFA, and the vast majority of drug-resistant mutations have been mapped to regions I, II and III and the additional region, A (Fig. 1). Mutations involving reduced susceptibility to nucleoside analogues and pyrophosphate analogues should reflect regions of the enzyme that are important for the binding of dNTP and pyrophosphate. Regions II and III contain the greatest clustering of mutations and are therefore considered most likely to interact directly with drugs and natural ligands (Coen, 1996a). HSV-1 strains that were selected for resistance against either PFA, PMEA or PMEDAP share a single amino acid change (Ser-724 to Asn) in conserved region II of the DNA polymerase. Furthermore, this amino acid change has pre-
Previously been shown to confer resistance to PFA (Larder et al., 1987; Gibbs et al., 1988). Therefore, our results indicate that the PME compounds interact at a site on the DNA polymerase that overlaps with the pyrophosphate-binding site. In contrast, HPMPA\textsuperscript{R} and HPMPC\textsuperscript{R} strains were found to contain mutations either in non-conserved regions or in non-conserved positions within conserved regions of the viral DNA polymerase. The significant mutations found in the HPMPC\textsuperscript{R} clones (i.e. excluding Ala-136 to Thr) are located within δ-region C (Val-573 to Met) and within region II (Arg-700 to Met). Based on the structure of the DNA polymerase from bacteriophage RB69 (Wang et al., 1997), residue 573 appears to be located within an α-helix in the 3′ → 5′ exonuclease editing domain. It is one turn of the helix before Tyr-577 and two turns before Asp-581, both of which are catalytically important. Thus, position 573 is very close to the catalytic site of this domain. Interestingly, position 700 is not conserved among DNA polymerases but an Arg-700 to Gly change has been reported in a PFA\textsuperscript{R} strain (Gibbs et al., 1988). The bacteriophage RB69 structure further suggests that position 700 is located within a β-strand (named β14) adjacent to the strands bearing the catalytic triad of Asp residues (717, 886 and 888 in HSV-1). Mutations at this position may affect the positioning of the base moiety of the incoming nucleotide.

The product of the UL42 gene is an accessory protein of HSV polymerase, a DNA-binding protein with an apparent molecular mass of 65 kDa, which functions as a processivity factor. The carboxy-terminal 35 amino acids of HSV DNA polymerase have been shown to be crucial for UL42-binding activity (Digard et al., 1993). The mutations in the HPMPA\textsuperscript{R} clones mapped to residues 1007 and 1028, beyond the last region of conserved residues in the catalytic domain and upstream the UL42-binding domain. On the basis of weak sequence similarity to the bacteriophage RB69 polymerase (Wang et al., 1997), we suggest that these residues are in the thumb subdomain of the catalytic domain. Our sequence alignment against the crystal structure suggests that the nearby (positively charged) Arg residues at positions 1019, 1020, 1026 and 1039 may play a role in binding the phosphate backbone of the duplex DNA product.

The different PMEA\textsuperscript{R}, PMEDAP\textsuperscript{R} and PFA\textsuperscript{R} clones were not significantly impaired in their ability to kill mice after intracerebral inoculation. This finding is in agreement with previous findings indicating that different drug-resistant DNA polymerase mutants may vary in their neurovirulence capacity (Field & Darby, 1980; Field & Coen, 1986; Sacks et al., 1989; Snoek et al., 1994; Coen, 1996a; Pelosi et al., 1998a). In contrast, the HPMPC\textsuperscript{R} clones were much less neurovirulent for mice than the wild-type KOS strain, suggesting that the molecular basis for drug-resistance at the level of the DNA polymerase may affect the neurovirulence of the mutants. In the case of the HPMPA\textsuperscript{R} isolates, the clones B5 and A3, with the Ile-1028 to Thr mutation, showed reduced neurovirulence compared with the wild-type KOS strain. However, the HPMPA\textsuperscript{R} clone D1, with the Leu-1007 to Met mutation and the HPMPA\textsuperscript{R} clone F1, which did not present mutations at position 1007 or 1028, had neurovirulence for mice similar to that of the parental KOS strain.

HSV mutants that are resistant to antiviral drugs due to mutations in the TK and/or DNA polymerase genes are an increasingly serious problem for immunocompromised patients (Safrin, 1996). The vast majority of HSV mutants that are

### Table 3. Phenotypic and genotypic characterization of various HPMPC\textsuperscript{R} and HPMPA\textsuperscript{R} HSV-1 clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>ACV</th>
<th>PFA</th>
<th>PAA</th>
<th>PMEA</th>
<th>PMEDAP</th>
<th>HPMPC</th>
<th>HPMPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} (µg/ml) mean ± SD</td>
<td>Amino acid change at position</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPMPC\textsuperscript{R} clones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clone D</td>
<td>0.013 ± 0.003</td>
<td>5.6 ± 3.8</td>
<td>2.1 ± 0.9</td>
<td>6.1 ± 3.4</td>
<td>4.3 ± 2.5</td>
<td>1.8 ± 1.2</td>
<td>1.0 ± 1.1</td>
</tr>
<tr>
<td>Clone H</td>
<td>0.008 ± 0.003</td>
<td>10.1 ± 1.8</td>
<td>7.7 ± 2.0</td>
<td>7.2 ± 1.9</td>
<td>3.1 ± 1.6</td>
<td>1.3 ± 0.6</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>Clone I</td>
<td>0.032 ± 0.019</td>
<td>7.6 ± 0.9</td>
<td>9.6 ± 2.0</td>
<td>10.0 ± 2.3</td>
<td>4.2 ± 2.5</td>
<td>2.5 ± 1.0</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>Clone M</td>
<td>0.010 ± 0.009</td>
<td>9.1 ± 0.8</td>
<td>2.9 ± 0.5</td>
<td>7.43 ± 3.60</td>
<td>3.17 ± 0.2</td>
<td>3.7 ± 2.9</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Clone E</td>
<td>0.018 ± 0.004</td>
<td>25.0 ± 4.4</td>
<td>10.3 ± 0.6</td>
<td>10.9 ± 3.7</td>
<td>2.8 ± 0.2</td>
<td>1.6 ± 0.7</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Clone J</td>
<td>0.020 ± 0.012</td>
<td>16.0 ± 5.6</td>
<td>10.3 ± 1.2</td>
<td>7.8 ± 2.1</td>
<td>2.1 ± 0.8</td>
<td>1.0 ± 0.5</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Clone N</td>
<td>0.022 ± 0.012</td>
<td>27.0 ± 5.3</td>
<td>10.5 ± 2.6</td>
<td>7.9 ± 3.8</td>
<td>2.7 ± 1.4</td>
<td>2.9 ± 1.7</td>
<td>2.4 ± 1.4</td>
</tr>
<tr>
<td>HPMPA\textsuperscript{R} clones</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Clone A3</td>
<td>0.026 ± 0.021</td>
<td>23 ± 5.5</td>
<td>8.0 ± 0.7</td>
<td>9.5 ± 1.7</td>
<td>3.4 ± 0.5</td>
<td>1.7 ± 1.0</td>
<td>1.7 ± 1.0</td>
</tr>
<tr>
<td>Clone F1</td>
<td>0.035 ± 0.026</td>
<td>27 ± 12</td>
<td>11.0 ± 1.0</td>
<td>15 ± 7</td>
<td>6.7 ± 3.9</td>
<td>2.7 ± 0.4</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Clone G</td>
<td>0.018 ± 0.009</td>
<td>39 ± 3</td>
<td>11 ± 1.6</td>
<td>10.2 ± 2.0</td>
<td>5.6 ± 1.6</td>
<td>2.1 ± 1.1</td>
<td>1.38 ± 0.54</td>
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</table>
Fig. 1. Mutations in the HSV-1 DNA polymerase. Locations of functional sites on the HSV-1 DNA polymerase and linear N terminus to C terminus configuration of the polypeptide are shown. Solid boxes show regions numbered I to VII on the basis of conservation among the DNA polymerase genes and region A, a region showing slight conservation. The locations of point mutations that result in altered drug sensitivity of the HSV-1 DNA polymerase are shown in the lower part of the figure. Previously reported laboratory and clinical mutants resistant to ACV and/or PFA are shown by thin arrows and the mutants reported here are indicated by thick arrows. References: 1, Larder et al. (1987); 2, Gibbs et al. (1988); 3, Tsurumi et al. (1987); 4, Knopf & Weisshart (1988); 5, Hwang et al. (1992); 6, Marcy et al. (1990); 7, Collins et al. (1989); 8, Chiou et al. (1995). The one letter amino acid code is used for the previously reported mutations.

attenuated in neurovirulence exhibit defects in replication in brain and/or in the peripheral nervous system (PNS) (Chou et al., 1990; Pyles et al., 1992). However, one DNA polymerase mutant, which is ACV\textsuperscript{r} due to an Arg-842 to Ser mutation in the conserved region III of the DNA polymerase, has recently been reported to be attenuated in neurovirulence, although it is able to replicate in the PNS and in the brain similarly to wild-type virus (Pelosi et al., 1998). Understanding how the different DNA polymerase mutations attenuate neurovirulence specifically may help to reveal the potential mechanism(s) of HSV pathogenesis.

The fact that selection of HSV-1 strains resistant to PME and HPMP derivatives is associated with different mutations at the level of the viral DNA polymerase has important consequences for the treatment of drug-resistant mutants that could arise during therapy with ANP analogues or with other drugs (e.g., ACV and PFA), emphasizing the importance of monitoring the drug susceptibility profile of isolates from patients with clinical drug resistance. Although selection of HSV-1 strains resistant to HPMPC has not been described in the clinic, it is important to know their pattern of sensitivity and their neurovirulence. The long-lasting antiviral effect of ANP derivatives, a remarkable feature of this class of compounds, allows infrequent administration of HPMPC in the treatment of HSV infections, thus reducing the probability of selection of HPMPC\textsuperscript{r} strains (Hitchcock et al., 1996; Naesens et al., 1997). We have demonstrated the usefulness of HPMPC in the treatment of ACV\textsuperscript{r}/PFA\textsuperscript{r} HSV infections, as well as the feasibility of alternating ACV and HPMPC therapy for the treatment of alternating ACV\textsuperscript{s} (ACV-sensitive) and ACV\textsuperscript{r} HSV infections in immunocompromised patients, since, as a rule, recurrences following HPMPC therapy show a reversion of the ACV\textsuperscript{r} to the ACV\textsuperscript{s} phenotype (Snoeck et al., 1994).

An HSV-2 isolate with reduced susceptibility to HPMPC selected in vitro has been reported by Mendel et al. (1997). This resistant virus had a reduced susceptibility to HPMPC but no change in its susceptibility to ACV and PFA. Notably, this strain proved to be severely compromised in its virulence for mice. Genotypic analysis of this HPMPC\textsuperscript{r} HSV-2 identified a single mutation (Gly-506 to Ser) located between region IV and region A within the proposed catalytic domain of the DNA polymerase.

It should be noted that multi-step drug selection of HSV-1 with PFA, PMEA and PMEDAP resulted in a homogeneous virus population, since all the different clones presented the same phenotypic and genotypic profile. In contrast, selection with HPMPC or HPMPA resulted in a heterogeneous virus population, since different amino acid changes that conferred resistance to HPMPC and HPMPA were identified, indicating that different regions of the HSV DNA polymerase may be involved in the interaction with these compounds. However, mutations in the DNA polymerase gene occur with low
frequency due to the fact that there exists only a restricted number of sites in the DNA polymerase gene at which changes may occur that result in drug resistance while maintaining a functional DNA polymerase. The cloning and sequencing of the DNA polymerase genes of the HPMPC<sup>r</sup> and HPMPA<sup>r</sup> clones that were negative for the mutations identified here is currently in progress. Marker transfer experiments to confirm that the polymerase mutations described in the present study confer the drug-resistance phenotype are also in progress. In addition, the possibility that mutations in other HSV DNA replication genes may play a role should be investigated further. This may be relevant to the HPMPC<sup>r</sup> isolates (clones C3 and A4) that showed hypersensitivity to the pyrophosphate analogues, since mutations in the viral major DNA-binding protein gene leading to altered substrate specificity.

In conclusion, HSV-1 strains resistant to HPMP derivatives are distinct from those that are resistant to PME derivatives. Furthermore, in contrast to the ACV<sup>r</sup>, PFA<sup>r</sup> and PME<sup>r</sup> mutations, the mutations arising under selective pressure of HPMP and HPMPA are unique and occur in non-conserved positions of the viral DNA polymerase. This has important consequences for antiviral chemotherapy, as it may form a scientific basis for the use of HPMP in the treatment of ACV<sup>r</sup> and/or PFA<sup>r</sup> HSV infections, as well as for the avoidance of selection of multi-drug-resistant virus in vivo. Since the (neuro)virulence of the virus strains seems to be associated with the type of drug resistance, mutation monitoring seems to be even more advisable.

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