The anti-HIV drug tenofovir, a reverse transcriptase inhibitor, also targets the herpes simplex virus (HSV) DNA polymerase

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Running title: Tenofovir targets the HSV DNA polymerase

Short summary: The anti-retroviral nucleotide analogues tenofovir and PMEO-DAPy target the HSV DNA polymerase as demonstrated by characterization of drug-resistant viruses and clinical isolates with DNA polymerase mutations emerging under acyclovir and/or foscarnet therapy showed cross-resistance to tenofovir and PMEO-DAPy.

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Abstract

Background. Genital herpes is an important cofactor for acquisition of HIV infection and effective prophylaxis is a helpful strategy to halt both HIV and HSV transmission. The antiretroviral agent tenofovir, formulated as a vaginal microbicide gel, was shown to reduce the risk of HIV and HSV-2 acquisition.

Methods. HSV-1 and HSV-2 mutants were selected for resistance to tenofovir and PMEO-DAPy (an acyclic nucleoside phosphonate with dual anti-HSV and anti-HIV activity) by stepwise dose escalation. Several plaque-purified viruses were characterized phenotypically (drug-resistance profiling) and genotypically (sequencing of the viral DNA polymerase gene).

Results. Tenofovir-resistant and PMEO-DAPy-resistant viruses harboured specific amino acid substitutions associated with resistance not only to tenofovir and PMEO-DAPy but also to acyclovir and foscarnet. These amino acid changes [A719V, S724N and L802F (HSV-1) and M789T and A724V (HSV-2)] were also found in clinical isolates recovered from patients refractory to acyclovir and/or foscarnet therapy or in lab-derived strains. A total of 10 (HSV-1) and 18 (HSV-2) well-characterized DNA polymerase mutants had decreased susceptibility to tenofovir and PMEO-DAPy.

Conclusions. Tenofovir and PMEO-DAPy target the HSV DNA polymerase and clinical isolates with DNA polymerase mutations emerging under acyclovir and/or foscarnet therapy showed cross-resistance to tenofovir and PMEO-DAPy.

Keywords: Tenofovir; PMEO-DAPy; DNA polymerase; herpes simplex virus; HIV; drug-resistance, PrEP

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BACKGROUND

Herpes simplex virus (HSV) is a common cause of both genital and oral diseases. HSV-2 is mostly sexually transmitted while HSV-1 is frequently acquired during early childhood, mainly via oral secretions. However, the epidemiology of HSV is changing, with increased frequency of HSV-1 sexual transmission [1-3]. Genital herpes is an important cofactor for HIV acquisition and preexposure prophylaxis (PrEP) is a helpful strategy to halt both HIV and HSV transmission [4-6].

The acyclic nucleoside phosphonate (ANP) tenofovir (TFV), an effective inhibitor of HIV reverse transcriptase, is one of the most commonly used antiretroviral drugs administered orally under its prodrug form tenofovir disoproxil fumarate (TDF) alone or in fixed-dose combinations. Recently, a newer prodrug of TFV (tenofovir alafenamide, TAF), with greater antiviral activity and better distribution into lymphoid tissues than TDF was approved [7].

TFV has also been evaluated for prevention of HIV and HSV-2 infections. The CAPRISA 004 study (first trial to evaluate TFV as a microbicide [8]), showed that a 1% TFV vaginal microbicide gel provided an overall protective effect of 39% against HIV acquisition and 51% against HSV-2 infection [8, 9]. However, the VOICE study that evaluated oral TFV alone and in combination with emtricitabine and TFV vaginal gel, was stopped early due to inability to demonstrate efficacy [10]. This negative outcome was mainly explained by the low adherence [11]. The FACTS 001 trial showed that pericoital vaginal TFV 1% gel was not effective in preventing HIV acquisition. An impact of oral TFV on HSV-2 or HSV-1 shedding rates was not observed in asymptomatic individuals co-infected with HIV and HSV [12]. The PEARLS A5175 study showed that HSV-2 acquisition was not reduced in HIV-infected/ HSV-uninfected adults treated with TDF as part of combination antiretroviral therapy [13]. However, daily oral TFV and emtricitabine-TFV PrEP reduced HIV and HSV-2 acquisition among heterosexual HIV-1 uninfected men and women (who were at risk for HIV-1 acquisition due to having an HIV-1-infected partner) [14, 15].

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The inconsistent results from clinical protocols using TFV-based regimens in PrEP have been primarily explained by poor adherence. Though inadequate drug concentrations at the site of infection (the genital tract) as well as integrity of the vaginal epithelium may be involved [11, 16]. Differences in TFV activation in peripheral blood mononuclear cells, vaginal and colorectal tissues due to variance in nucleotide kinase expression and activity were reported [17, 18]. Furthermore, genetic variants of the kinases that phosphorylate TFV could affect its activation, influencing drug efficacy.

TFV has been known as a highly potent antiretroviral drug with minimal anti-HSV activity [19]. However, TFV proved direct anti-herpetic activity at the concentration achieved intravaginal with a 1% TFV topical gel [16]. Topical application of 1% TFV gel on the genital mucosa resulted in TFV concentrations of up to 350 µg/ml in vaginal fluid, which is above the 50% effective concentration (EC_{50}) against both HSV-1 and HSV-2 [20]. Furthermore, the active TFV metabolite (TFV-diphosphate) inhibited both HSV DNA polymerase (pol) and HIV reverse-transcriptase activities in enzymatic assays [20]. However, because of the poor activity of TFV against HSV, concerns have been raised regarding its selectivity and specificity as an anti-HSV agent.

In an effort to develop a compound highly potent against both HIV and HSV-2, we identified PMEO-DAPy (6-phosphonylmethoxyethoxy-2,4-diaminopyrimidine) [21]. This compound demonstrated potent anti-HIV and -HSV activity in clinically relevant *in vitro*, *ex vivo*, and *in vivo* systems. However, in striking contrast to TFV, PMEO-DAPy also acts as an efficient immunomodulator without the need of intracellular metabolism [21].

To prove definitively that TFV and PMEO-DAPy target the HSV DNA pol, we have now selected and characterized phenotypically and genotypically viral mutants emerging under pressure with both drugs. In addition, well-characterized lab- or clinic-derived DNA pol mutants have been evaluated for their sensitivity to TFV and PMEO-DAPy.

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METHODS

Cells and viruses.

Human embryonic lung (HEL) fibroblasts (ATCC CCL-137) were maintained in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum. HEL cells were used for production of viral stocks, selection of drug-resistant viruses and antiviral assays.

The HSV-1 reference strain Kos (ATCC VR-1493) and the HSV-2 reference strain G (ATCC VR-734) were used.

Isolation of drug-resistant viruses.

HEL cells were seeded in 25-cm^2 flasks and infected with the reference HSV strains in the presence of TFV or PMEO-DAPy starting at a concentration equivalent to the EC_{50}. For HSV-2, mutants were also selected under pressure with 2-phosphonylmethoxyethyl (PME) derivatives of adenine (PMEA, adefovir) and 2,6-diaminopurine (PMEDAP). When full cytopathic effect (CPE) was reached, samples were frozen and the virus was harvested and used to infect cells for the next passage. This process was repeated several times in increasing concentrations of the compound. Each drug-resistant virus was passaged two or three times in the highest drug concentration [TFV 400 µg/ml, PMEA 200 µg/ml, PMEDAP and PMEO-DAPy 50 µg/ml] and further cultured for one additional passage in drug-free medium. Several drug-resistant clones were then isolated and tested for their drug-sensitivity.

Antiviral assays.

The drug susceptibilities of the different virus clones were determined by CPE reduction assays in HEL cell cultures as previously described [22, 23]. For each viral mutant, at least 3 to 6 independent phenotypic assays were performed and the mean EC_{50} values were calculated. HSV isolates were considered resistant at ≥2-fold increase in EC_{50}.

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Genotypic antiviral resistance testing.

Viral DNA was extracted from clinical isolates and laboratory-derived strains or plaque-purified viruses by using a QIAamp DNA blood mini kit (Qiagen). A set of eight M13-labeled primers were used for gene amplification of the full-length UL30 (DNA pol) gene (3,708 bp for HSV-1 and 3,723 bp for HSV-2). PCR was performed using the proofreading Faststart High Fidelity PCR System kit (Sigma) and amplified products were purified with the QIAquick PCR Purification Kit (Qiagen). The amplicons were sequenced with the BigDye Terminator v3.1 sequencing kit (ThermoFischer Scientific) and analyzed with the automated sequencer ABI 3730 genetic analyzer (Applied Biosystems). All nucleotide and amino acid sequencing results were compared with the reference strains Kos (HSV-1) and G (HSV-2) using Seqscape version 2.7 software.

RESULTS

Genotyping of drug-resistant TFV and PMEO-DAPy viruses compared to other anti-HSV drugs and relationship with previously described HSV DNA pol mutations.

To undoubtedly determine that the HSV DNA pol is the target of TFV, we selected TFV-resistant (TFV-R) HSV-1 and HSV-2 mutants and genotyped the UL30 gene. Three (HSV-1) and two (HSV-2) independent procedures of selection with TFV were performed. All HSV-1 TFV-R plaque purified viruses harbored the L802F amino acid change in the viral DNA pol while all HSV-2 TFV-R clones had the A724V mutation (Table 1). The HSV-1 DNA pol L802F change has been previously described in viruses selected *in vitro* for PMEA-resistance [22] while the A724V substitution has been associated with acyclovir/foscarnet-resistance both *in vitro* and in the clinic [24, 25].

HSV-1 PMEO-DAPy-resistant (PMEO-DAPy-R) viruses presented the A719V (1 clone) or the S724N (4 clones) substitutions. Both mutations have been reported in clinical strains associated with failure to acyclovir and foscarnet treatment [26, 27] and have been selected *in vitro* under pressure with foscarnet (A719V and S724N)

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and the PME derivatives (S724N) [22, 23]. In the case of HSV-2 PMEO-DAPy-R viruses, all four clones presented the M789T.

Because, no data were available for HSV-2 PMEA-R and PMEDAP-R viruses, we genotyped clones isolated following pressure with these two drugs (Table 1). Similar to PMEO-DAPy-R viruses, all HSV-2 clones selected under PMEA had the M789T substitution. Two different substitutions (M789T, A724V) associated with drug-resistance were identified in PMEDAP-R clones. Figure 1 shows the location of the mutations emerging under the PME derivatives compared to those arising under TFV and PMEO-DAPy.

Phenotyping of drug-resistant TFV and PMEO-DAPy viruses.

We then examined the drug-susceptibility profile of TFV-R and PMEO-DAPy-R viruses bearing specific DNA pol mutations. The HSV-1 L802F, A719V, and S724N and the HSV-2 A724V and M789T were associated with TFV-R when using a cutoff value of ≥ 2-fold change in EC_{50} values (Figure 2). The highest levels of resistance to TFV were measured for the HSV-1 S724N and A719V mutants (≥5-fold increase in EC_{50} values) and for the HSV-2 A724V mutant (≥4.4-fold increase in EC_{50} value). The HSV-2 M789T and the HSV-1 L802F substitutions conferred, respectively, ≥ 2.3- and ≥ 3.7-fold resistance to TFV. These mutants were also resistant to PMEO-DAPy (3.7- to 22.5-fold increase in EC_{50} value), the PME derivatives and foscarnet. A 3.2- to 4.5-fold decrease in susceptibility to acyclovir was measured for the TFV-R and PMEO-DAPy-R viruses and no cross-resistance with brivudine or ganciclovir was registered. In the case of penciclovir and cidofovir, no cross-resistance or low levels of resistance were measured [1.6- to 2.6-folds (penciclovir) and of 1.6- to 2.8-folds (cidofovir) increase in EC_{50} values].

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Relevant TFV and PMEO-DAPy resistance mutations exist in the clinic.

We have previously reported that thymidine kinase (TK) HSV-1 and HSV-2 mutants remained sensitive to both tenofovir and PMEO-DAPy [20, 21]. We have now evaluated several clinical strains (recovered from patients that failed anti-herpesvirus therapy with acyclovir and/or foscarnet in the context of our translational research platform RegaVir, www.regavir.org) that bear alterations in the viral DNA pol gene. Well-characterized clinical isolates of HSV-1 (5) and HSV-2 (18) with specific substitutions in the viral DNA pol as well as 7 laboratory-derived strains (6 for HSV-1 and 1 for HSV-2) were assessed (Tables 2 and 3). All DNA pol mutants proved resistant to TFV and PMEO-DAPy and to the PME derivatives. Except for the HSV-1 Q618H, and the N815S (homologous to the HSV-2 N820S) mutants, cross-resistance with the pyrophosphate analogue foscarnet was found. While all tested DNA pol mutants proved also acyclovir-R, not all of them had decreased sensitivity to penciclovir. Importantly, the HSV-1 N815S mutant and the corresponding HSV-2 N820S showed the highest levels of resistance to acyclovir and penciclovir and were the only mutants presenting low levels of ganciclovir-resistance. Strikingly, these mutants demonstrated hypersensitivity to foscarnet.

With the exception of the S724N and L802F (HSV-1) and the I731F (HSV-2) mutants that had a 2.3- to 2.8-fold decrease in EC_{50} for cidofovir, all other mutants remained sensitive to this nucleotide analogue. Data for brivudine are only provided for HSV-1 as this compound exhibits very low potency against HSV-2 due to a lack of conversion of the monophosphate-brivudine to the diphosphate form by the HSV-2 TK, which is deprived deoxythymidylate kinase (dTMP) activity [28]. All evaluated HSV-1 DNA pol mutants remained sensitive to brivudine.

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Analysis of DNA pol mutants.

The HSV DNA pol (Figure 1) is a multi-functional enzyme of 1235 (HSV-1) and 1240 (HSV-2) amino acids. It possesses polymerase activity (for extension of DNA primer chains), an intrinsic 3'-5' exonuclease activity (important for correction of misincorporated nucleotides and maintenance of the fidelity and integrity of the newly formed DNA molecules), and 5'-3' exonuclease and ribonuclease (RNase) H activities [29, 30]. The carboxyl terminal region of the UL30 catalytic subunit interacts with the UL42 accessory protein, which enhances the processivity of DNA replication.

The HSV DNA pol belongs to the B family DNA pols and is composed of six structural domains: pre-NH_2, NH_2, palm, fingers, and thumb domains as well as a 3'→5' exonuclease domain. The HSV DNA pols share seven clusters of homology conserved among all α-like DNA pols that are numbered in order of decreased percentage of homology (regions I to VII). In addition, a δ-region C, which is homologous to eukaryotic DNA pols δ is found in herpesvirus DNA pols. The regions I, II and VII are part of the palm subdomain and together with region V flank the catalytic site that contain the three aspartic acid residues (at positions 717, 886 and 888) that are essential for pol activity. Regions III and VI play a role in positioning the template and primer strands.

Structure of the active RB69 bacteriophage DNA pol complexed to its substrates (pdb code: 4DU1) was used to build a model of HSV DNA pol in complex with (R)-PMPA-diphosphate, PMEO-DAPy-diphosphate, acyclovir-triphosphate or foscarnet. Located in the conserved region II of the Palm domain, the HSV-1 A719V change and its counterpart A724V in HSV-2, may impair the correct binding of both (R)-PMPA-diphosphate and PMEO-DAPy-diphosphate at the position of the γ-phosphate (Figure 3). This can be explained by the bulkier side chain of the valine residue (surface: 155 $Å^2$, volume: 140 $Å^3$) in comparison with the alanine residue (surface: 115 $Å^2$, volume: 88.6 $Å^3$) modifying the shell of residues surrounding the active site. Similarly, the HSV-1 S724 is also located in the conserved region II of the Palm domain, just below the A719 position. Substitution of a serine residue

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(surface: 115 Å^2, volume: 89 Å^3) by an asparagine residue (surface: 160 Å^2, volume: 114.1 Å^3) might affect the positioning of A719 among other residues involved directly in the interaction with the nucleoside analogue and therefore the correct binding of PMEO-DAPγ-diphosphate. It is worth noting that A719V and S724N in HSV-1 DNA pol and A724V in HSV-2 DNA pol confer also resistance to foscarnet. The position A719 (A724 in HSV-2 DNA pol) and S724 in HSV-1 DNA pol are close to the β- and γ-phosphates of the incoming nucleoside triphosphate, a position that is competitively adopted by foscarnet.

Asparagine 815 (N815) in HSV-1 DNA pol is located in the Finger domain and participates to the interaction with the incoming nucleoside triphosphate. The N815S substitution confers resistance to acyclovir and ganciclovir and hypersensitivity to foscarnet, which may be explained by a better stabilization of foscarnet in the region that binds the pyrophosphate analogue. While N815 interacts with foscarnet indirectly through hydrogen bonds with a water molecule, the substitution by a serine residue reduces the volume of the side chain at position 815, allowing foscarnet to adopt a preferential position between the Fingers and the Palm domains. Figure 4A shows the change in steric hindrance that may occur when the asparagine (yellow dots) is substituted by a serine (red dots) at position 815. In contrast, this should have the opposite effect on acyclovir binding since it may reduce the interactions with the phosphates via water molecules (Figure 4B).

DISCUSSION

Genital herpes, a highly prevalent sexually transmitted disease, is recognized as one of the most common causes of genital ulcers in developed and developing countries. Infection of ectocervical tissue with HSV-2 results in mucosal immune cell activation increasing the frequency of $CD4^+$ cells [4]. Because infection with HSV-2 has been associated with an increased risk for HIV-1 infection and often worsen the clinical outcome of HIV disease [29, 30], strategies to prevent transmission of both HIV-1 and HSV are highly desirable.

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TFV is considered as one of the leading HIV microbicide candidate. PMEO-DAPy, another ANP with dual anti-HSV and anti-HIV activity, may be considered as a second-line microbicide candidate. By selecting TFV and PMEO-DAPy resistant viruses, we have now clearly demonstrated that both drugs indeed target the HSV DNA pol explaining their anti-HSV activity. Mutant viruses selected under pressure with these nucleotide analogues were resistant not only to TFV and PMEO-DAPy but also to foscarnet and acyclovir. Ten (HSV-1) and eighteen (HSV-2) DNA pol substitutions, including fourteen novel HSV-2 amino acid changes, could be linked to TFV and PMEO-DAPy, with some mutations showing specific drug-resistance patterns. Phenotypic analysis of novel HSV DNA pol mutations is crucial to guide therapeutic options in the clinic. Although most of the HSV acyclovir-R infections are due to alterations in the viral enzyme responsible for its activation (i.e. TK), a substantial number of DNA pol mutations are found in isolates recovered from patients that fail acyclovir therapy and in individuals that receive foscarnet therapy for acyclovir-R infections due to TK alterations [27, 31-33].

Mutations emerging under TFV and PMEO-DAPy selective pressure mapped to region II of the viral DNA pol with exception of L802F (located between regions VI and III). These amino acid substitutions were identical to those emerging under pressure with the PME derivatives adefovir and PMEDAP, suggesting that these ANPs interact in a similar manner with the viral enzyme. The greatest number of known mutations conferring drug-resistance in HSV DNA pol cluster in the conserved regions II, III, VI and VII that are directly or indirectly involved in recognition and binding of nucleotides or pyrophosphate and in catalysis [29, 34]. Most of the HSV DNA pol substitutions here reported were found in viruses that remain sensitive to cidofovir. However, the HSV-1 S724N and L802F changes and the HSV-2 I731F substitution were associated with low levels of resistance to cidofovir (2- to 2.8-fold reduced EC_{50}'s). Therapeutic options in these cases are quite limited although these viruses retained sensitivity to brivudine and ganciclovir, which represent alternative therapies if the virus still carries a wild-type TK.

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Enzymatic assays demonstrated that the active form of the acyclic pyrimidine nucleoside phosphonate PMEO-DAPy, i.e. PMEO-DAPy-diphosphate, is recognized by the HIV-1 reverse transcriptase as a purine (e.g. adenine) nucleotide derivative but not as a pyrimidine [35]. Modeling and docking studies in HIV reverse transcriptase confirmed that PMEO-DAPy-diphosphate fits into the active site of the HIV-1 reverse transcriptase in a similar manner as TFV-diphosphate mimicking an incomplete purine ring. Investigation of the activity of PMEO-DAPy-diphosphate against reverse transcriptases containing the K65R and K70E mutations (that are associated, respectively, with TFV and PMEA resistance) [36, 37] indicated that both mutations conferred resistance to PMEO-DAPy-diphosphate via a similar mechanism, i.e. reduced catalytic efficiency of incorporation [35]. In the present study, we demonstrated using HSV genotypic and phenotypic assays and molecular modeling that PMEO-DAPy, TFV and the PME derivatives are recognized by the HSV DNA pol similarly.

HSV drug-resistance is associated with severe, debilitating mucosal disease and visceral dissemination, constituting an important clinical problem for immunocompromised patients. Among immunocompetent individuals, a low prevalence (range from 0.1% to 0.6%) of acyclovir-R has been reported [24]. In contrast, a prevalence of HSV acyclovir-R isolates of up to 36% was found in hematopoietic stem cell transplant recipients [38] and ranging from 3.5% to 7% in HIV patients [24]. While HSV infection in the immunocompetent host commonly requires short-term anti-HSV therapy, without development of drug-resistance, patients with an impaired immune system generally require long-term anti-HSV therapy to manage persistent infections being prone to develop drug-resistance infections. Also, and most important, when the host responses are impaired, less pathogenic viruses that would not survive under normal host responses may continue to replicate. Different degrees of pathogenicity in animal models of HSV infection have been described among TK and DNA pol mutants [22, 24, 39].

Emergence of TFV-R HIV strains isolated in the clinic is rare and is mostly associated with the reverse transcriptase K65R/N and K70E/G/Q changes [36, 37]. However, extensive TFV-R has been reported to emerge

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after virological failure in a high proportion of patients undergoing a TFV-containing first-line regimen among low-income and middle-income regions in the world [40]. The spectrum of TFV-selected mutations appeared to extend beyond K65R/N and K70E/G/Q as several additional TFV-associated mutations of unknown clinical significance were identified [41]. In our studies, we demonstrated that several substitutions in the HSV DNA pol are associated with TFV-R even if only the DNA mutations L802F (HSV-1) and A724V (HSV-2) changes emerged under TFV pressure.

The use of TFV in combination with a HSV-specific topical antiviral agent to deliver a better prevention of HSV acquisition has not yet been investigated [42]. However, a major drawback in the development of microbicides is poor adherence. To improve delivery systems that minimize the difficulties linked to adherence, intravaginal rings that deliver TFV and the prodrug TDF are currently in early stages of clinical development [43].

Taking into account that the target of all approved anti-HSV drugs is the DNA pol and that a single mutation in this viral enzyme may confer resistance to many anti-HSV drugs, there is an urgent need to develop novel classes of anti-HSV drugs. The helicase-primase inhibitor ASP2151 (amenamivir) possesses activity against HSV and varicella-zoster virus (VZV) [44]. Because of adverse findings in a phase 1 trial, the development of amenamivir was suspended, though clinical development has meanwhile been resumed in Japan for HSV (and VZV) patients with multiple orofacial or genital lesions [45]. Another helicase-primase inhibitor, pritelivir (AIC316, BAY 57-1293), discovered by Bayer [46], is currently in clinical development by AiCuris. In a phase 2 clinical trial, pritelivir met the primary endpoint by being more effective for suppression of viral shedding compared to valacyclovir, the current standard treatment for genital herpes [47]. Further research is required to assess longer-term efficacy and safety of the drug as well as emergence of resistance following long-term administration. If the safety and efficacy of these helicase-primase inhibitors is confirmed, these drugs could be used not only for the treatment of HSV TK and/or DNA pol mutants but also in PrEP in combination with anti-HIV agents.

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In conclusion, our studies clearly demonstrated that TFV and PMEO-DAPy specifically target the HSV DNA pol considering that single substitutions in this viral enzyme were associated with decreased sensitivity to these drugs and more importantly, the mutant viruses emerging under TFV and PMEO-DAPy presented alterations in the HSV DNA pol.

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Conflicts of interest

The authors declare no conflicts of interest.

Legends to the Figures

Figure 1. Diagram of the HSV-1 and HSV-2 DNA pols.

Figure 2. Resistance properties of plaque-purified HSV-1 and HSV-2 clones selected following pressure with TFV and PMEO-DAPy which bear substitutions in the viral DNA pol. Different plaque-purified viruses bearing specific mutations in the DNA pol genes were compared with wild-type virus using the cytopathic effect (CPE) reduction assay. At least three independent experiments were performed for each plaque-purified virus. Each individual point represents the mean values from independent experiments for one particular plaque-purified virus. The data are presented as a dot plot of the 50% effective concentration (EC_{50}) for the drug-resistant

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clones versus the EC_{50} for the wild-type virus. Average (indicated as a horizontal line) ± standard errors (bars) are indicated for the different types of mutants and the wild-type virus. TFV was tested at a maximum concentration of 200 µg/ml and the levels of resistance are expressed as ≥ than a certain value when the EC_{50}'s for the drug were in some antiviral assays higher than the maximum concentration evaluated. The sources of the compounds used in the antiviral assays were as follows: acyclovir [ACV, 9-(2-hydroxyethoxymethyl)guanine], GlaxoSmithKline, Stevenage, United Kingdom; foscarnet [PFA, foscavir, phosphonoformate sodium salt] and penciclovir [PCV, 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine], Sigma Chemicals, St Louis, MO; tenofovir [TFV, (*R*)-PMPA, (*R*)-9-(2-phosphonylmethoxy-propyl)adenine]; adefovir {ADV, PMEA, 9-[2-(phophonylmethoxyethyl)adenine]} and cidofovir {CDV, (*S*)-HPMPC , (S)-1-[3-hydroxy-2-(phosphonylmethoxypropyl)cytosine]}, Gilead Sciences, Foster City, CA; PMEDAP {9-[2-(phosphonylmethoxy-ethyl)-2,6-diaminopurine] and PMEO-DAPy {6-[3-hydroxy-2-(phosphonomethoxy)ethoxy]-2,4-diaminopyrimidine}, Dr Marcela Krecmerova, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic]; brivudine [(E)-5-(2-bromovinyl)-1-(β-D-2'-deoxyribofuranos-1)-yl-uracil, BVDU], Searle, United Kingdom; and ganciclovir [GCV, 9-(1,3-dihydroxy-2-propoxymethyl)guanine], Roche, Basel, Switzerland.

Figure 3. Models of mutant UL30 DNA pols in complex with PMEO-DAPy-diphosphate [PMEO-DAPy-DP] (A) and (*R*)-PMPA-diphosphate [(*R*)-PMPA-DP] (B). Substitutions A719V in HSV-1 UL30 (A724V in HSV-2 UL30) are represented by yellow (alanine residue) and red dots (valine residue). The colored dots depict the changes in volume of the amino acids side chain. Substitution S724N in HSV-1 UL30 is represented as red stick. The model was built using the structure of RB69 bacteriophage DNA pol (pdb code: 4DU1) and the images generated using PyMol Delano software.

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Figure 4. Models of mutant UL30 DNA pols in complex with foscarnet (FOS) (A) and acyclo-GMP (acyclovir monophosphate) (B). N815S is represented as yellow dots (N815) and red dots (S815) showing the shift in side chain volume that occupied each residue. The model was built using the structure of RB69 bacteriophage DNA pol (pdb code: 4DU1) and the images generated using PyMol Delano software.

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Table 1. DNA pol mutations in PMEO-DAPy and (R)-PMPA (TFV)-resistant HSV-1 & HSV-2 clones as well as HSV-2 mutants selected under PMEA and PMEDAP pressure.

Virus	Compound used for selection	Clone	DNA pol substitutions associated with:		Phenotyping performed
			Natural genetic polymorphisms linked to inter-strain variability	Drug-resistance	
HSV-1	PMEO-DAPy	Clone 1	G33S – T566A – K700R	A719V	+
		Clone 2	G33S – T566A – K700R	S724N	+
		Clone 3	G33S – T566A – K700R	S724N	+
		Clone 4	G33S – T566A – K700R	S724N	+
		Clone 5	G33S – T566A – K700R	S724N	+
	(*R*)-PMPA (TFV)	Clone A-5	G33S – T566A – K700R	L802F	+
		Clone B-1	G33S – T566A – K700R	L802F	+
		Clone B-2	G33S – T566A – K700R	L802F	+
		Clone C-1	G33S – T566A – K700R	L802F	+
		Clone C-2	G33S – T566A – K700R	L802F	+
HSV-2	PMEO-DAPy	Clone 1	A9T – E678G – P801T – G904A – E905A – A906G	M789T	+
		Clone 2	A9T – E678G – P801T – G904A – E905A – A906G	M789T	+
		Clone 3	E678G – P801T – G904A – E905A – A906G	M789T	+
		Clone 4	E678G – P801T – G904A – E905A – A906G	M789T	+
		Clone 5	E678G – P801T – G904A – E905A – A906G	M789T	+
		Clone 6	E678G – P801T – G904A – E905A – A906G	M789T	+
HSV-2	(*R*)-PMPA (TFV)	Clone A-1	P801T – G904A – E905A – A906G	A724V	+
		Clone A-2	A9T – P801T – G904A – E905A – A906G	A724V	+
		Clone C-1	A9T – P801T – G904A – E905A – A906G	A724V	-
		Clone C-2	P801T – G904A – E905A – A906G	A724V	-
		Clone C-3	P801T – G904A – E905A – A906G	A724V	+
		Clone C-4	P801T – G904A – E905A – A906G	A724V	+
		Clone C-5	P801T – G904A – E905A – A906G	A724V	-
HSV-2	PMEA	Clone C	A9T – E678G – P801T – G904A – E905A – A906G	M789T	+
		Clone D	A9T – E678G – P801T – G904A – E905A – A906G	M789T	+
		Clone E	E678G – P801T – G904A – E905A – A906G	M789T	+
		Clone F	A9T – A435V – E678G – P801T – G904A – E905A – A906G	M789T	+
	PMEDAP	Clone B	A435V – E678G – P801T – G904A – E905A – A906G	A724V	+
		Clone C	A9T – A435V – E678G – P801T – G904A – E905A – A906G	A724V	+
		Clone D	R265H – E678G – P801T – G904A – E905A – A906G – A915T*	none	+
		Clone E	A9T – E678G – P801T – G904A – E905A –A906G A906G	M789T	+

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* Novel amino acid change not associated with drug-resistance.
For tenofovir, 2 (HSV-2) or 3 (HSV-1) independent procedures of selection (designated A, B and C) were performed. For each selection procedure, 1 (HSV-1 clone A-5), 2 (HSV-1: clones B-1 & B-2 and clones C-1 & C-2; HSV-2: clones A-1 & A-2) and 5 (HSV-2: clones C-1, C-2, C-3, C-4, & C-5) clones were analyzed. Thus, the L802F HSV-1 and the HSV-2 A724V DNA pol substitutions independently emerged 3 and 2 times, respectively. In the case of PMEA, PMEDAP and PMEO-DAPy, a single selection procedure was performed and then several clones were analyzed.

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Table 2. Sensitivity of well-characterized HSV-1 DNA pol mutant viruses

Mutant virus	Strain origin	Location in the HSV DNA pol (enzyme subdomain)	Amino acid substitution in HSV-1 (HSV-2)*	Fold-resistance (Ratio EC_{50} mutant / EC_{50} Kos strain)**									
				ACV	PCV	BVDU	GCV	PFA	ADV	PMEDAP	TFV	PMEO-DAPy	CDV
PMEA-R (clone 1055)	Lab	δ-region C (3'→5' exonuclease)	Q618H	**3.4**	**2.9**	0.3	0.9	1.0	**≥5.0**	**>11.6**	**≥2.8**	**≥9.4**	0.6
RV-301	Clinic		A605V (A606V)*	**6.1**	1.2	0.2	0.6	**6.7**	**≥2.5**	**≥8.7**	**>3.0**	**>7.9**	1.6
PFA-R (clone 1022)	Lab	Region II (Palm)	V714M	**2.1**	**3.6**	1.1	0.9	**2.3**	**2.2**	**3.5**	**≥2.3**	**2.3**	1.0
RV-255	Clinic		A719V (A724V)*	**7.0**	**2.8**	0.5	1.6	**3.6**	**3.2**	**6.5**	**≥3.0**	**≥7.9**	0.7
RV-312	Clinic		A719T (A724T)*	**5.4**	1.4	1.0	1.8	**2.1**	**≥2.5**	**3.1**	**≥2.2**	**2.7**	1.0
PFA-R (clone C)	Lab		S724N (S729N)*	**4.1**	**6.6**	0.4	0.9	**3.1**	**>5.0**	**6.3**	**≥2.8**	**≥11.5**	**2.8**
RV-HU-5	Clinic		S724N (S729N)*	**8.6**	**2.3**	0.2	1.9	**4.9**	**>4**	**7.1**	**>3.0**	**>7.9**	**2.6**
PMEA-R (clone 1005)	Lab	Between regions VI and III (Fingers)	L802F	**2.4**	**2.8**	0.9	0.7	**2.2**	**≥3.0**	**3.8**	**≥2.8**	**3.7**	**2.2**
RV-189	Clinic	Region III (Fingers)	N815S (N820S)*	**77.8**	**13.2**	0.6	**3.7**	0.3	**>4.0**	**10.7**	**>3.0**	**≥7.4**	1.1
PFA-R (clone 1034)	Lab		T821M	**3.3**	**2.9**	1.0	0.9	**2.6**	**≥2.9**	**5.6**	**≥2.3**	**5.6**	1.5
PMEA-R (clone 157A)	Lab	Region V (Thumb)	R959H (R954H)*	**3.3**	**2.6**	1.0	0.9	**3.5**	**≥4.6**	**9.0**	**≥2.8**	**≥11.5**	1.8

***** Homologous mutation in HSV-2 linked to drug-resistance. ** Fold-resistance levels ≥2-fold are highlighted in bold. The amino acid substitutions that were also found in viral clones derived from selective pressure with TFV or PMEO-DAPy are highlighted in grey. Abbreviations: ACV (acyclovir), PCV (penciclovir), BVDU (brivudine), GCV (ganciclovir), PFA (foscarnet), ADV (adefovir, PMEA), TFV (tenofovir), CDV (cidofovir). TFV, ADV, PMEDAP and PMEO-DAPy were tested at a maximum concentration of 200 µg/ml (TFV and ADF) or 50 µg/ml (PMEDAP and PMEO-DAPy) and the levels of resistance are expressed for some of the mutants as ≥ than a certain value when the EC_{50}'s for the drugs were in some antiviral assays higher than the maximum concentration evaluated.

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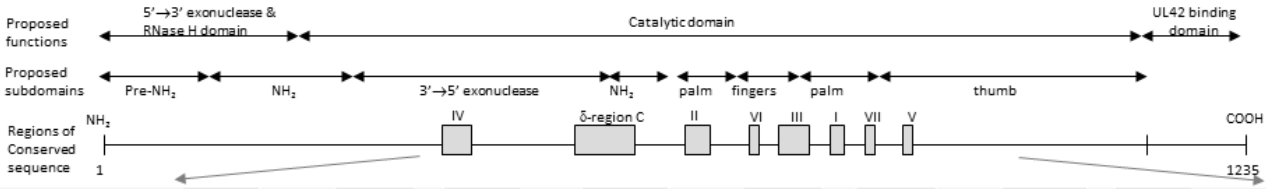
Table 3. Sensitivity of well-characterized clinical HSV-2 DNA pol mutants

Mutant virus	Strain origin	Location in the HSV DNA pol (enzyme subdomain)	Amino acid substitution in HSV-2 (HSV-1)	Fold-resistance**								
				ACV	PCV	GCV	PFA	ADV	PMEDAP	TFV	PMEO-DAPy	CDV
RV-197 clones 31 & 32	Clinical	3'→5' exonuclease	K533E (K532T)*	**3.8**	**2.5**	1.0	**4.2**	**3.2**	**5.0**	**≥3.0**	**5.2**	1.4
RV-204 clones 1 to 4	Clinical	δ-region C (3'→5' exonuclease)	A606V (A605V)*	**6.2**	**2.6**	0.8	**6.2**	**10**	**8.7**	**≥3.0**	**12.7**	1.7
RV-197 clones 4, 33 & 39	Clinical		C625R	**3.3**	**3.4**	1.4	**4.4**	**6.1**	**3.4**	**2.9**	**3.8**	1.4
RV-197 clones 1, 30 & 40	Clinical		R628C	**2.0**	**2.2**	0.9	**2.5**	**3.7**	**2.7**	**2.0**	**4.7**	1.3
RV-200 clones 3 & 9	Clinical		R628C	**2.0**	1.8	0.6	**2.4**	**2.1**	**2.6**	**≥2.3**	**5.0**	1.2
RV-203 clones 19, 23 & 25	Clinical	Region II (Palm)	A724V (A719V)*	**3.6**	1.6	0.5	**2.7**	**2.4**	**3.5**	**2.8**	**4.7**	1.0
RV-203 clones 1 to 6	Clinical		S725G	**2.7**	1.7	0.5	**4.0**	**3.6**	**2.9**	**2.2**	**4.6**	0.7
RV-206 clone 6	Clinical		S729N (S724N)*	**6.9**	**3.8**	1.2	**6.9**	**9.7**	**7.7**	**3.7**	**21**	1.6
RV-201 clones 1, 2 & 4	Clinical		I731F	**2.9**	1.7	0.8	**6.0**	**3.6**	**5.0**	**≥2.4**	**11.6**	**2.3**
RV-202 clones 3 & 5	Clinical		Q732R	**3.8**	**2.7**	0.8	**4.1**	**4.3**	**3.4**	**≥2.5**	**5.5**	1.8
RV-206 clones 1, 2, 3, 4 & 6	Clinical	Region VI (Fingers)	M789T	**3.3**	**2.7**	0.7	**3.2**	**4.0**	**5.3**	**≥2.5**	**11.3**	1.4
RV-202 clone 4	Clinical		M789K	**3.4**	**2.7**	0.6	**3.3**	**3.1**	**5.8**	**≥3.4**	**13.6**	1.6
RV-228	Clinical	Region III (Fingers)	V818A (V813A)*	**4.6**	**2.8**	0.9	**5.1**	**≥7.7**	**7.3**	**≥3.3**	**≥3.2**	1.1
RV-1024	Clinical		N820S (N815S)*	**68**	**5.9**	**4.0**	0.2	**≥15.3**	**≥5.5**	**≥3.3**	**≥6.2**	1.0
RV-204 clones 5, 19, 21 & 23	Clinical		Y823C	**3.4**	1.6	0.6	**2.0**	**2.6**	**2.5**	**≥2.6**	**4.8**	1.3
PFA^{R} (clones 1 to 7)	Lab		R847C	**6.6**	**2.4**	0.8	**6.1**	**5.9**	**6.7**	**≥4.7**	**8.0**	1.6
RV-200 clones 1, 2 & 6	Clinical	Between regions I and VII (Palm)	F923L	**7.5**	**3.2**	0.8	**4.7**	**4.5**	**6.7**	**≥3.6**	**11.4**	1.4
RV-196 clones 1 to 6	Clinical		T934A	**5.0**	**2.8**	0.8	**5.4**	**4.8**	**5.8**	**>3.1**	**6.9**	1.1
RV-197 clone 2	Clinical	Region V	R964H (R959H)*	**4.9**	1.2	0.4	**4.8**	**7.6**	**8.3**	**3.3**	**26.3**	1.0

* Homologous mutation in HSV-1 linked to drug-resistance. ** Fold-resistance levels ≥2-fold are highlighted in bold. The amino acid substitutions that were also found in viral clones derived from selective pressure with TFV or PMEO-DAPy are highlighted in grey. Underlined substitutions indicate previously undescribed mutations. Abbreviations: ACV (acyclovir), PCV (penciclovir), GCV (ganciclovir), PFA (foscarnet), ADV (adefovir, PMEA), TFV (tenofovir), CDV (cidofovir). TFV, ADV, PMEDAP and PMEO-DAPy were tested at a maximum concentration of 200 µg/ml (TFV and ADF) or 50 µg/ml (PMEDAP and PMEO-DAPy) and the levels of resistance are expressed for some of the mutants as ≥ than a certain value when the EC_{50}'s for the drugs were in some antiviral assays higher than the maximum concentration evaluated.

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Figure 1



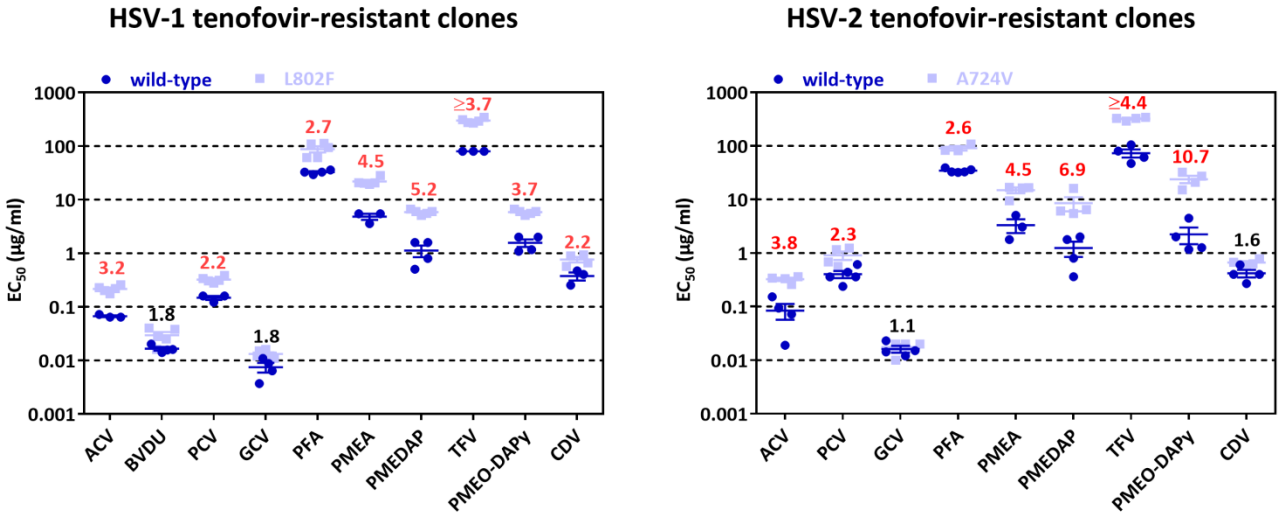
HSV-1	Region IV 437-479		δ-region C 577-637	Region II 694-736	Region VI 772-791		Region III 805-845	Region I 881-896		Region VII 938-946	Region V 953-963
Mutations isolated *in vitro* under pressure with adefovir (PMEA) and PMEDAP			Q618H	S724N S724N		L802F					R959H
Mutations isolated *in vitro* under pressure with PMEO-DAPy and tenofovir				A719V S724N		L802F					
Other mutations here described			A605V	V714M			N815M T821M				

HSV-2	Region IV 438-480		δ-region C 578-638	Region II 699-741	Region VI 777-796		Region III 810-850	Region I 886-901		Region VII 943-951	Region V 954-968
Mutations isolated *in vitro* under pressure with adefovir (PMEA) and PMEDAP				A724V	M789T M789T						
Mutations isolated *in vitro* under pressure with PMEO-DAPy and tenofovir				A724V	M789T						
Other mutations here described		K533E	A606V C625R R628C	S725G S729N I731F Q732R	M789K		V818A N820S Y823C R847C		F923L T934A		R964H

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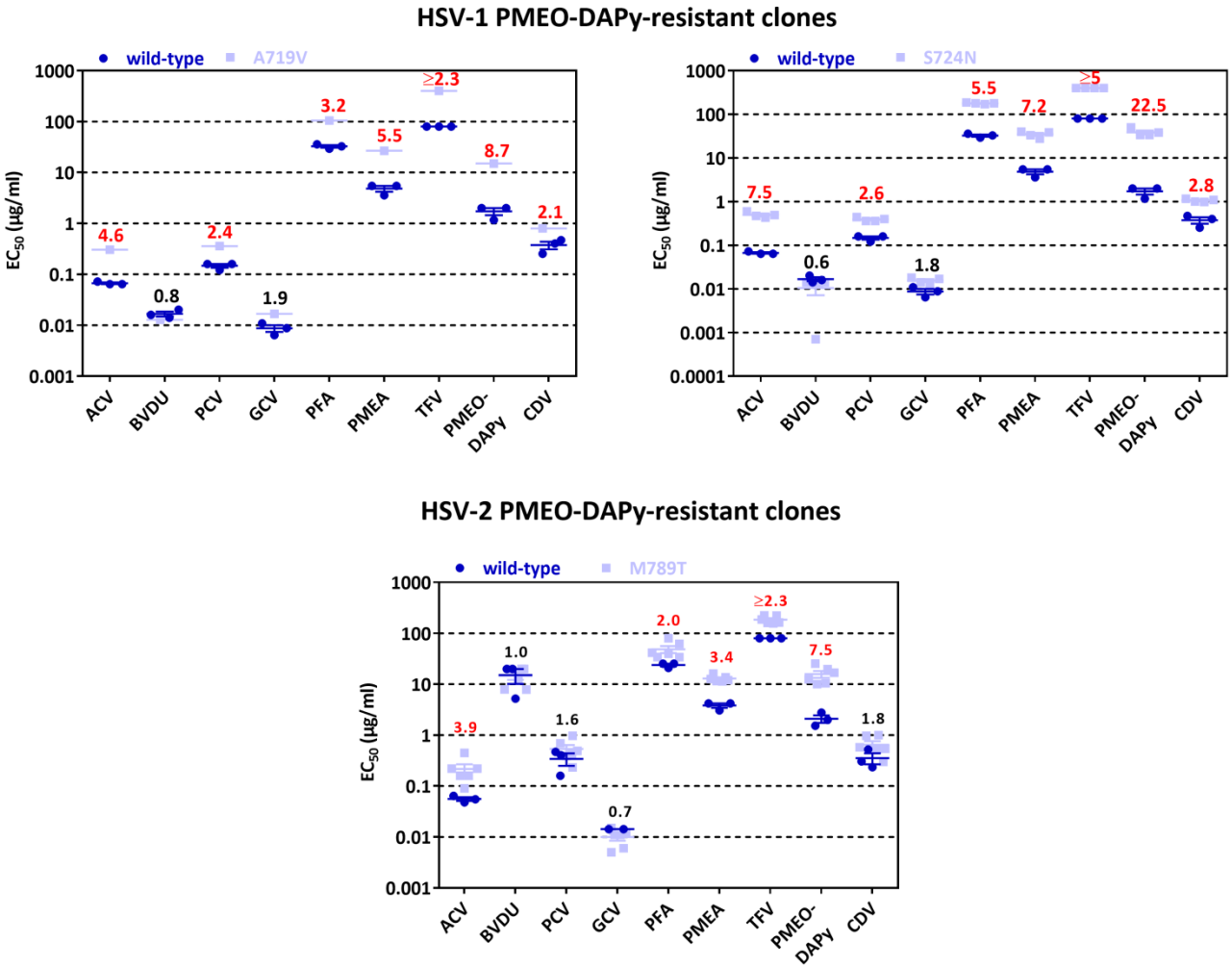
Figure 2A



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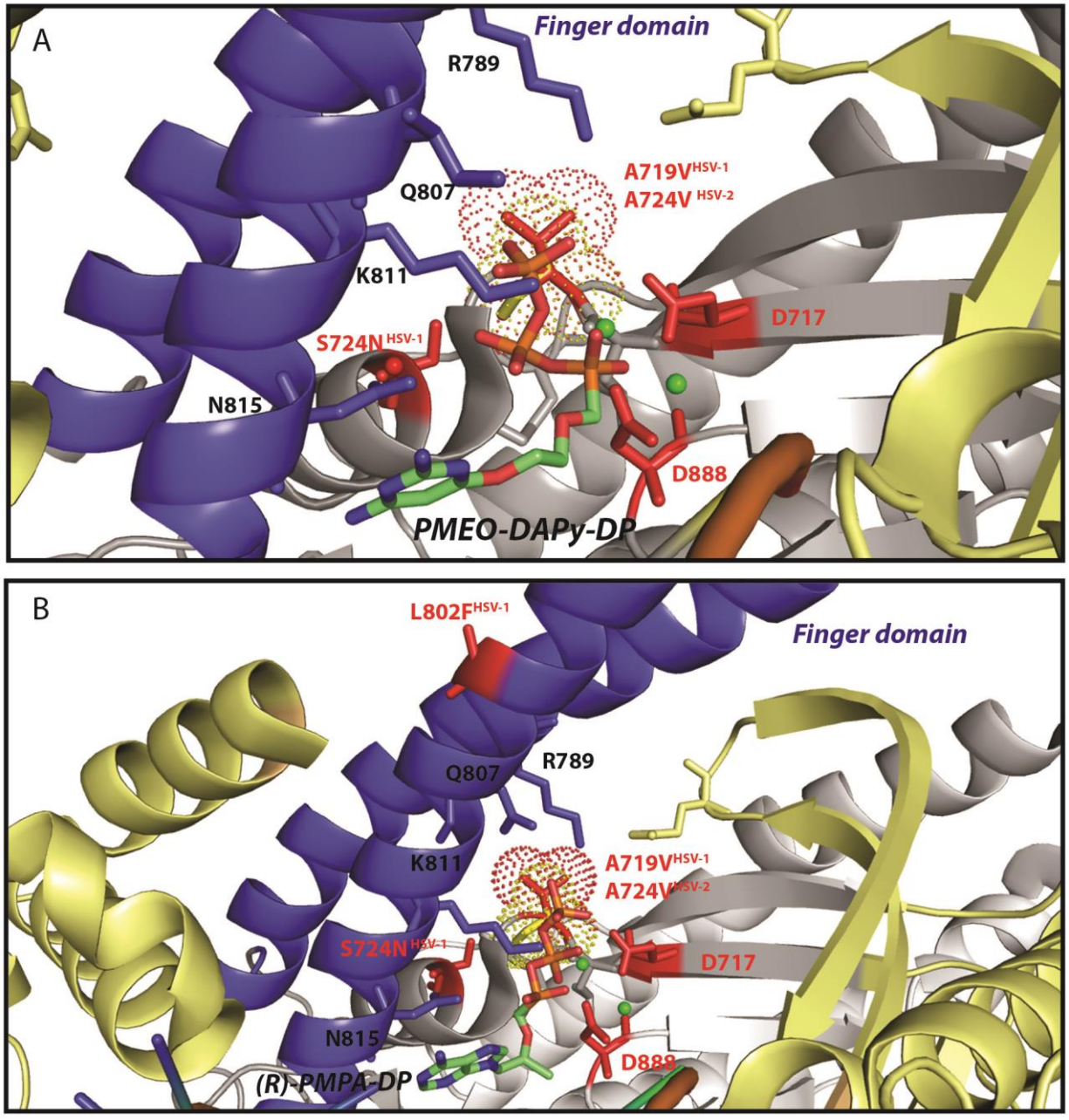
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Figure 2B



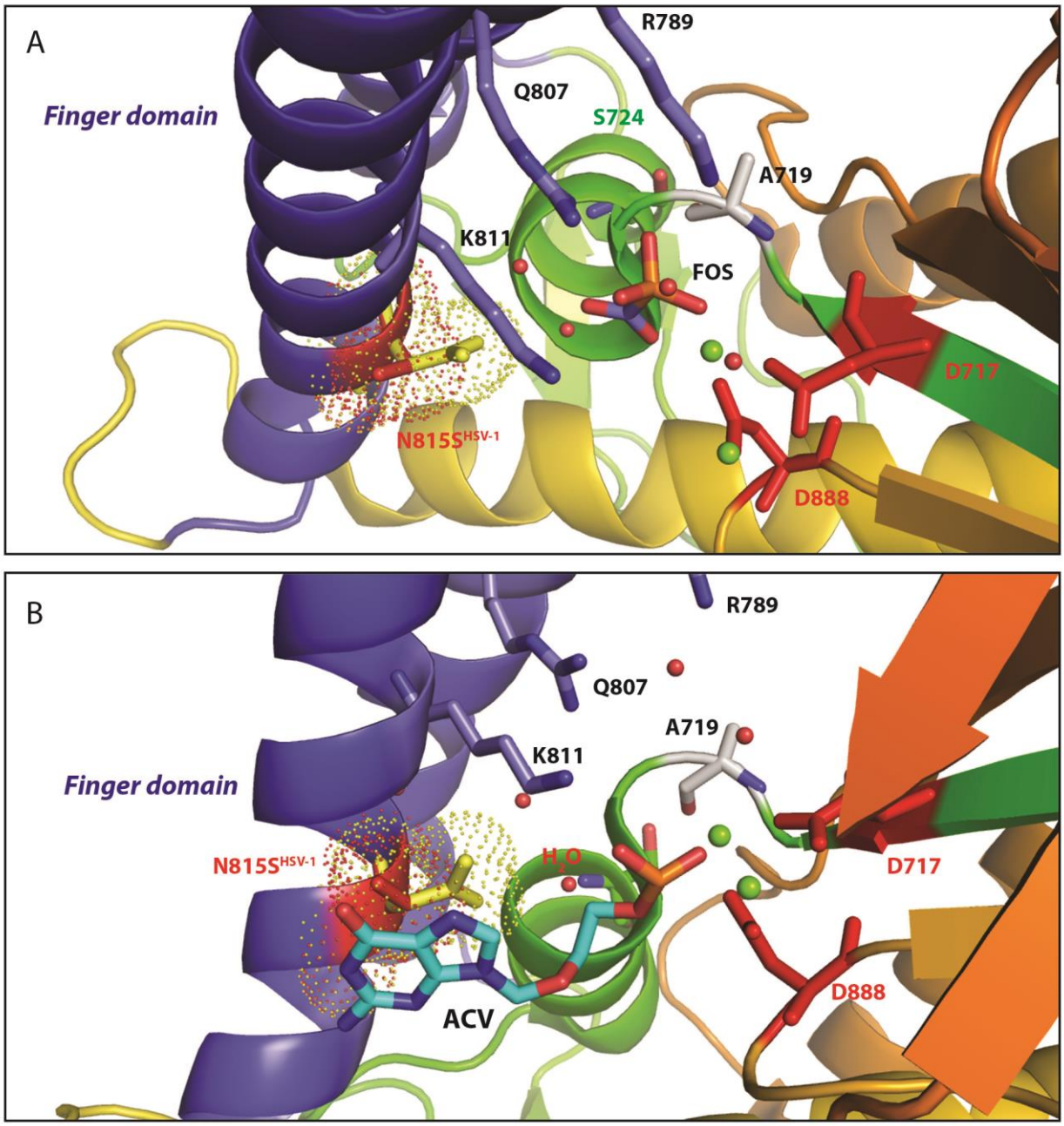
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Figure 3



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Figure 4



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