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E17A mutation in HIV-1 Vpr confers resistance to didanosine in association with thymidine analog mutations

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ABSTRACT

Background: HIV-1 accessory Vpr protein is involved in the reverse transcription process and has been shown to modulate the virus mutation rate. This process may play a role in the kinetics of appearance of drug resistance mutations under antiretroviral treatment.
Methods: Vpr sequences were analyzed from plasma viruses derived from 97 HIV-1-infected individuals failing antiretroviral treatment and 63 antiretroviral-naïve patients. Vpr genetic variability was analyzed for association with specific drug treatment and drug resistance mutations. Biological and virological experiments were employed to characterize a mutation in Vpr found to be associated with virological failure.
Results: E17A mutation located in the first α-helix of Vpr was more prevalent in HAART-treated individuals compared to untreated individuals. E17A was associated with thymidine analog mutations (TAMs) in reverse transcriptase M41L, L210W and T215Y and with the use of didanosine in the patients' treatment histories. E17A had no impact on the biochemical and functional properties of Vpr, and did not affect kinetics of replication of wild-type or TAMs-containing viruses. However, its association with TAMs and the use of didanosine was consistent with phenotypic susceptibility assays showing a significant 3-fold decrease in didanosine susceptibility of viruses harboring Vpr E17A combined with TAMs compared to viruses harboring TAMs alone.
Conclusion: These findings highlight a novel role of Vpr in HIV-1 drug resistance. Vpr E17A confers resistance to didanosine when associated with TAMs. Whether Vpr E17A facilitates excision of didanosine is still to be determined.

1. Introduction

The high adaptive capacity of HIV-1 is demonstrated by its ability to escape from potent antiviral drugs in treated HIV-infected individuals. Virological failure occurs when viruses with drug resistance-associated mutations (RAMs) in the targeted viral regions (reverse transcriptase, protease, integrase or envelope) are selected and outgrow the more susceptible strains. The generation of resistant variants is a consequence of the error proneness of the HIV-1 reverse transcriptase (RT) enzyme (Mansky and Temin, 1995) and recombination (Jetzt et al., 2000; Levy et al., 2004; Moutouh et al., 1996). In addition to *gag*, *pol* and *env* genes, HIV-1 genome encodes a number of accessory proteins that may be involved in viral diversification and generation of resistant variants. For example, Vif and Vpr have been shown to be indirectly involved in the reverse transcription process (Bishop et al., 2004; Mansky and Temin, 1995).

We recently demonstrated that some Vif variants are likely involved in the appearance of drug resistance-associated mutations

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in vivo. Patients harboring viral strains encoding partially active Vif proteins are more likely to develop resistance to certain antiretroviral agents (ARV) as these strains are able to promote the selection of some G-to-A substitutions leading to drug-resistance mutations (Fourati et al., 2010). Another accessory protein, Vpr, has been shown to participate in the accuracy of the reverse transcription process in *in vitro* experiments. Vpr is a 96 amino acid protein that consists of a flexible N-terminal region, three α-helical domains with amphipathic properties and a flexible C-terminal region (Morellet et al., 2003). Vpr is incorporated into HIV-1 virions through direct interaction with the p6 domain of the Gag precursor (Bachand et al., 1999; Lavallee et al., 1994). This protein is a multifunctional protein and was associated with a variety of roles in determining HIV-1 infectivity including an effect on the reverse-transcription process, nuclear transport of the HIV-1 pre-integration complex, cell cycle arrest at the G2/M transition, induction of apoptosis and transactivation of the HIV-1 LTR (for reviews, see Andersen et al., 2008; Le Rouzic and Benichou, 2005). Some of these functions involve interactions with cellular partners, including viral and host–cell proteins. For example, disruption of cell-cycle control is consequent to Vpr interaction with the DCAF1 subunit of the Cul4a/DDB1 E3 ubiquitin ligase (Belzile et al., 2007; DeHart et al., 2007; Le Rouzic et al., 2007) and may provide a favorable environment for viral expression (Goh et al., 1998). Finally, the role of Vpr in the reverse transcription process implies interaction with the nuclear form of the DNA repair enzyme uracil DNA glycosylase (UNG2) for modulating the virus mutation rate (Chen et al., 2004; Mansky et al., 2000). Thus, this function of Vpr may play a role in kinetics of appearance of drug resistance-associated mutations. However, the *in vivo* significance of the above-mentioned process is unknown.

To our knowledge, there is no report evaluating the impact of primary Vpr variants from infected patients as a mechanism underlying resistance to ARVs. In the present study, we investigated Vpr genetic variability in antiretroviral-experienced patients failing highly active antiretroviral treatment (HAART) compared to a drug–naïve population. We identified one mutation E17A in Vpr found more frequently in HAART-treated patients. We studied the functional properties of this variant and evidenced a decreased susceptibility to didanosine of this variant when associated with TAMs in cell culture-based assays.

2. Methods

2.1. Patients

A total of 160 HIV-1 plasma viruses subtype B derived from infected patients (97 ARV-experienced failing HAART and 63 treatment naïve) were analyzed. In ARV-experienced subjects, HIV-1 genotypic resistance was initiated because of HAART failure. Resistance testing was performed by sequencing protease (PR) and reverse transcriptase (RT) regions following the French National consensus technique (http://www.hivfrenchresistance.org). Additionally, we analyzed 63 plasma samples from HIV-1 infected patients who were treatment-naïve and for whom HIV genotype testing was performed at the time of diagnosis.

2.2. Amplification and sequencing of Vpr gene

RNA isolation, cDNA synthesis, and PCRs were performed as described previously (Malet et al., 2008). For the first round PCR, we used Vpr1 (5′-gaagtacacatcccactagggga-3′) and VprB (5′-ctccgcttcttcctgccat-3′). The second-round PCR was performed using primer Vpr3 (5′-attggggtctgcatacaggagaaa-3′) and VprD (5′-gccataggagargcctaagcc-3′).

2.3. Site directed mutagenesis

pNL4-3 clones containing mutations in the RT (M41L, L210W, T215Y, K65R) and/or Vpr (E17A) coding regions were constructed by site-directed mutagenesis using the Quickchange II Site Directed mutagenesis Kit (Stratagene) according to the Manufacturer's instructions. All HIV-1 constructs were verified by nucleotide sequencing. (Primers: for the introduction of M41L, we used M41L-S 5′-agaaaaaataaaagcattagtagaaatttgtacagaact ggaaaaggaaggaaaa-att-3′ and M41L-AS 5′-aatttttccttccttttccagttctgtacaaatttctactaatgctttt attttttct-3′; for the introduction of L210W and T215Y, we used 1(210/215)S : 5′-gaggaactgag acaacatctgtggaggtggggattttacacaccagac-3′ and 2(210/215)AS: 5′-gtctggtgtgtaaaatc cccacctccacagatgttgtctcagttcctc-3′; for the introduction of K65R, we used 1-K65R-S: 5′-tactccagtatttgccataaagagaaaagacagtactaaatggagaa-3′ and 2-K65R-AS 5′-ttctccatttagt actgtcttttctctttatggcaaatactggagta-3′; for the introduction of Vpr E17A, we used 1vpr17S 5′-gggccacagagggagccatacaatgcatggacactagagc-3′ and 2vpr17AS 5′-gctctagtgtccatgcattgtatgg ctccctctgtggccc-3′).

2.4. Cell culture and transfection

HeLa and 293 T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 50 units/ml penicillin/streptomycin, and 125 ng/ml amphotericin B (Invitrogen), at 37 °C under 5% CO_2. For pulldown and co-immunoprecipitation experiments, 293T cells were transfected using Lipofectamine 2000 (Invitrogen), according to the Manufacturer's recommendations. For immunofluorescence experiments, HeLa cells were grown onto coverslips in 6-well plates and transfected using the GeneJuice method (Novagen), according to the Manufacturer's recommendations. For the virion packaging assay, cells were co-transfected as described using the calcium phosphate method (Jacquot et al., 2007). For the generation of HIV-1 stocks, 293 T cells were transfected with pNL4-3 plasmids using Lipofectamine Plus reagent (Invitrogen).

2.5. Assay for virion incorporation of Vpr-UNG2 fusions

Incorporation of the wild-type and mutated Vpr proteins into HIV-1 virions was analyzed as previously described (Chen et al., 2004), using a virion packaging assay in which HA-tagged Vpr were expressed in trans in virus-producing cells.

2.6. Immunofluorescence analysis

Plasmids for expression of HA-tagged form of the wild-type (WT) Vpr protein has been already described (Chen et al., 2004), while the plasmid for expression (E17A mutant) was constructed by PCR-mediated site-directed mutagenesis using specific primers containing the desired mutation as described (Chen et al., 2004). Twenty-four hours after transfection with vectors encoding either the WT or E17A HA-Vpr proteins or with the empty plasmid, cells were analyzed by indirect immunofluorescence as described previously (Langevin et al., 2009).

2.7. Recombinant proteins and GST pulldown assay

Plasmids for expression of HA-tagged forms of the wild-type and W54R Vpr proteins have been already described (Chen et al., 2004), while the plasmid for expression the E17A mutant was constructed by PCR-mediated site-directed mutagenesis using specific primers containing the desired mutation as described (Chen et al., 2004). Plasmids for bacterial expression UNG2 fused to the glutathione S-transferase (GST) has been also described (Chen et al., 2004). Recombinant GST and GST-UNG2 were produced in

Escherichia coli and then purified on glutathione (GSH)–Sepharose beads as described previously (Chen et al., 2004). Lysates from 293T cells previously transfected with plasmids for expression of wild-type or mutated HA-Vpr were incubated overnight at 4 °C with 2 μg of immobilized GST fusions as described (Chen et al., 2004). Bound proteins were separated by SDS–PAGE and analyzed by immunoblotting using the rat anti-HA monoclonal antibody (Roche Applied Science).

2.8. Co-immunoprecipitation assay

Plasmid for expression of UNG2 fused to the green fluorescent protein (GFP-UNG2) was kindly provided by Geir Slupphaug (Norvegian University of Science and Technology, Trondheim, Norway). 293T cells were transfected with plasmids for expression of the wild-type or mutated HA-Vpr proteins in combination with the GFP-UNG2 expression plasmid. 24 h later, cells were suspended in lysis buffer containing 100 mM $(NH_4)_2SO_4$, 20 mM Tris (pH 7.5), 10% glycerol, 1% Igepal CA-630 and 1× complete protease inhibitor mixture (Roche Diagnostics GmbH). After 30-min incubation at 4 °C under gentle agitation, cell lysates were centrifuged at 14,000*g* for 30 min at 4 °C. HA-Vpr proteins were immunoprecipitated from cleared lysates containing 400 μg of total proteins with 2 μg of anti-GFP (Roche Applied Science) on protein A–Sepharose beads. 80% of the precipitated material was then resolved by SDS–PAGE and analyzed by Western blotting using anti-HA and anti-GFP antibodies (Roche Applied Science).

2.9. Phenotypic drug susceptibility assays

Single-cycle titers of the virus were determined in HeLa-P4 cells (Larder et al., 1990; Larder and Kemp, 1989; St Clair et al., 1991) by quantifying β-galactosidase activity as previously described (Delelis et al., 2009). Cells were infected with viruses and grown in the presence of increasing concentrations of didanosine or 3′-azido-3′-deoxythymidine (AZT), ranging from 0.01 μM to 100 μM. The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration giving 50% inhibition of β-galactosidase levels with respect to untreated infected cells. Resistance to didanosine is frequently associated with mutations L74 V or K65R in RT in patients samples. Many studies using site-directed mutagenesis or recombinant viruses have shown that the magnitude of IC50 increase of viruses harboring either L74 V or K65R was similar in regards to didanosine (Petropoulos et al., 2000; Winters et al., 1997). In this study, we tested a K65R mutant as a control in didanosine phenotypic susceptibility assays. Drug susceptibility results were expressed as the fold change in susceptibility, defined as the ratio of the IC_{50} of the mutant variant to that of NL4-3 wild-type. Assays were performed in triplicate.

2.10. Statistical analyses

Comparisons between percentages were performed using the Fisher exact test. We used the entropy program to evaluate the differences in polymorphisms between the drug-naïve and the antiretroviral-experienced population. A Bonferroni correction was considered to set the threshold of significance (http://www.hiv.lanl.gov/cgi-bin/ENTROPY). The 50% inhibitory concentration (IC_{50}) was determined using regression curves. Analyses were performed using Statview.

2.11. Nucleotide sequence accession number

All the HIV-1 Vpr sequences related to this work have been submitted to GenBank and were given accession numbers JN125863 through JN126022.

3. Results

3.1. Patient characteristics

Our population of patients failing HAART ($n = 97$) has the following characteristics: the median age was 46 years, 89% of the patients were men; the median CD4 cell count was 331 cells/mm^3, and the median plasma HIV-1 RNA was 4680 cp/ml at the time of genotypic testing. Overall, median time of HIV-1 infection was 17 years (range: 4–25) and the median of prior ART exposure was 13 years (range: 1.5–18). Patients received a median of eight antiretroviral drugs in their history (range: 2–19). All patients received at least one nucleoside/nucleotide reverse transcriptase inhibitor (NRTI), 74% at least one non-nucleoside reverse transcriptase inhibitor (NNRTI) and 97% at least one protease inhibitor (PI) in their history. At the time of analysis, 98% of viruses collected from plasma from HAART-treated patients harbored at least one drug resistance associated mutation (RAM) in reverse transcriptase (RT) and/or protease (PR) (major mutations, as defined by the IAS-USA guidelines).

3.2. Vpr polymorphisms in patients failing ARVs and association with known ARV resistance associated mutations

A total of 160 HIV-1 plasma viruses subtype B were analyzed. The polymorphism within the HIV-1 Vpr primary sequence, deduced from sequencing of viral RNA from plasma samples from HAART-treated patients failing HAART ($n = 97$) was compared with the polymorphism from samples from therapy-naïve individuals ($n = 63$). Overall, the Vpr sequences were highly conserved in therapy-naïve patients with nearly 80% (74/97) of conserved amino acids (polymorphism <5%) in the Vpr coding region. In contrast, in HAART-treated patients, the Vpr proteins were more polymorphic with less than 50% amino acids being conserved (44/97). As reported in Fig. 1 and Table 1, E17, L22 and R32 positions located in the first α-helix of Vpr were more polymorphic in HAART-treated individuals ($p < 0.001$, $p = 0.02$ and $p = 0.02$, respectively). Specifically, E17A mutation increased in prevalence from 3% ($n = 2/63$) in samples from untreated individuals to 18% ($n = 17/97$) in samples from HAART-treated patients ($p = 0.004$) (Fig. 1, Table 1). R32K mutation increased in prevalence from 3% ($n = 2/63$) in samples from untreated individuals to 13% ($n = 13/97$) in samples from treated patients ($p = 0.02$).

To determine whether these two Vpr mutations were associated with any specific drug resistance-associated mutation, we analyzed protease and reverse transcriptase sequences derived from our patient database. We compared the prevalence of every drug resistance associated mutation in PR and RT as listed by the ANRS algorithm (http://www.hivfrenchresistance.org) from 17 patients harboring Vpr A17 with 70 patients harboring Vpr E17. Only three thymidine analog mutations (1) (TAMs-1) in the reverse transcriptase were found to be associated with E17A mutation: M41L ($p = 0.03$) L210W ($p = 0.006$) and T215Y ($p = 0.03$). In contrast, none of TAMs (2) including D67 N, K70R, T215F, and K219Q/E were associated with Vpr E17A. (D67 N, $p = 1$; K70R, $p = 0.53$; T215F, $p = 0.3$; K219Q/E, $p = 0.76$). Analyzing the individual drug regimens in HAART-treated patients, we observed a unique association between E17A and didanosine ($p = 0.03$) in the patients treatment histories (Fig. 2). Didanosine had been administered for a median of 2.5 years (range: 0.5–4.5). Similar analyses were performed for R32K mutation, but the Vpr R32K variant was not associated with a drug resistance mutation in PR or RT. Since no association of Vpr R32K was evidenced with either a specific drug in the patient treatment histories, or a specific drug resistance-associated mutation, we did not further characterize R32K mutation.

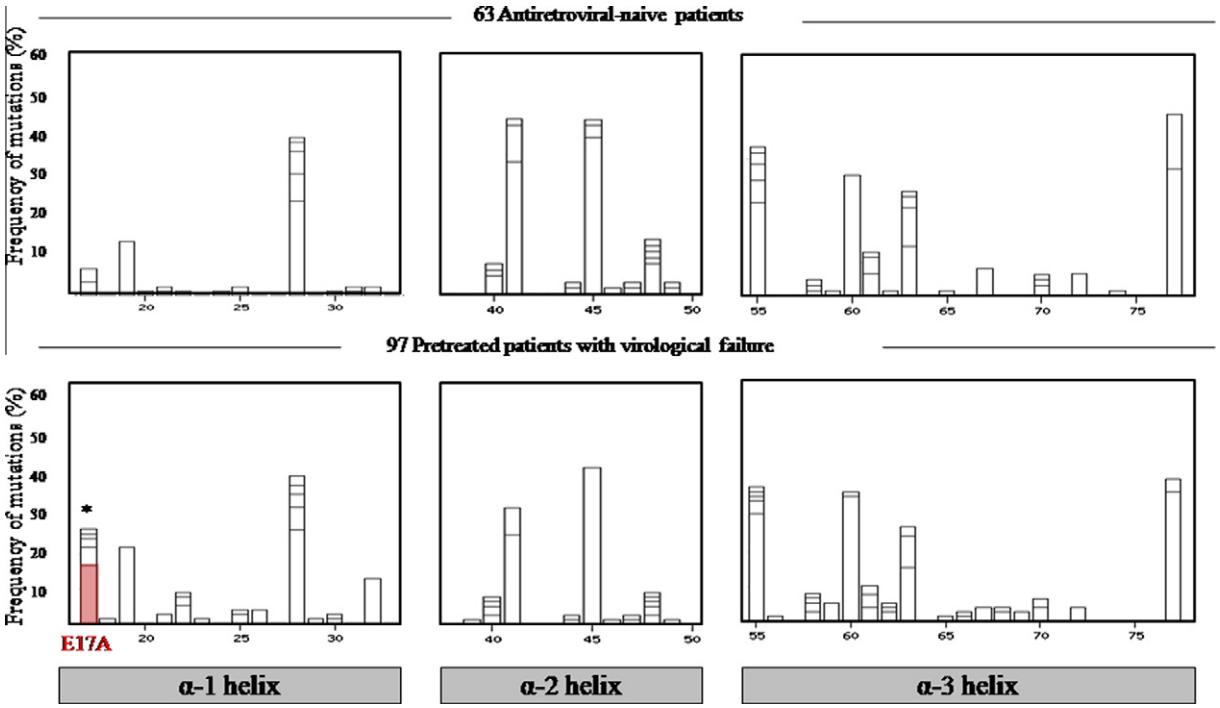

Fig. 1. Frequency of mutations in the three α-helix functional domains of HIV-1 Vpr. The amino acids variation shown is based on 63 sequences derived from drug-naïve patients and 97 sequences derived from patients failing on HAART. The variation in the three α-helix functional domains is shown for each of the two patient populations. Substitution at position 17 is indicated by *. HxB2 was used as reference sequence.

Table 1
Genetic variations in E17, L22 and R32 positions of the first α-helix of Vpr that were found more polymorphic in pretreated patients ($p < 0.05$, entropy program http://www.hiv.lanl.gov/cgi-bin/ENTROPY). E17A and R32K mutations were more prevalent in pretreated patients compared to untreated patients.

Position	Amino acid	No. (%) of patients	
		Pretreated	Untreated
17	E (wild type)	71 (73%)	59 (93%)
	A	**17 (18%)**	**2 (3%)**
	Q	4 (4%)	2 (3%)
	D	3 (3%)	0
	G	1 (1%)	0
	V	1 (1%)	0
22	L (wild type)	88 (91%)	62 (98%)
	F	4 (4%)	0
	I	4 (4%)	1 (2%)
	R	1 (1%)	0
32	R (wild type)	84 (86%)	61 (97%)
	K	**13 (14%)**	**2 (3%)**

3.3. Characterization of the Vpr E17A variant

Because we identified a significant association between the Vpr E17A mutation and failure to antiretroviral treatment in patients using didanosine, this substitution was first introduced within the Vpr protein of the HIV-1 NL4-3 isolate for biochemical and functional characterization.

Since the requirement of Vpr for early stages of the virus life cycle, including modulation of the reverse transcription process (for review, see Le Rouzic and Benichou, 2005), has been associated with its packaging into virions, we used a transient Vpr packaging assay in which HA-tagged Vpr was expressed in trans in virus-producing cells for analyzing whether the Vpr E17A mutant was incorporated into virus particles. As evidenced in Fig. 3A by western blotting, Vpr E17A was packaged into purified virions as efficiently as the wild-type Vpr protein. After *de novo* expression in infected cells, HIV-1 Vpr primarily localizes in the nucleus but it is excluded from the nucleolus (Depienne et al., 2000; Kamata and Aida, 2000). When expressed in HeLa cells as HA-tagged proteins and analyzed by immunofluorescence, both the wild-type and the Vpr E17A proteins were efficiently concentrated in the nucleus at steady state (Fig. 3B). Finally, the role of Vpr in the modulation of the virus mutation rate was related to its direct interaction with the nuclear form UNG2, a DNA repair enzyme involved in the cellular mechanisms of base-excision repair that specifically removes uracil from DNA (Planelles and Benichou, 2009). Therefore, we used biochemical approaches to investigate whether the Vpr E17A mutant retained the ability to interact with UNG2. An *in vitro* binding assay was used with recombinant UNG2 expressed in *E. coli* in fusion with the GST (GST-UNG2, see Fig. 3C, ponceau red). Purified GST-UNG2 was immobilized on GSH–Sepharose beads and then incubated with lysates from cells expressing wild-type or mutated HA-tagged Vpr proteins. Bound proteins were analyzed by immunoblotting with anti-HA (Fig. 3C, lower panels). Both wild-type and E17A HA-Vpr specifically bound to GST-UNG2 but not to GST alone. As a control, the VprW54R mutant, a well-characterized Vpr variant that fails to interact with UNG2 (Chen et al., 2004), was not retained on GST-UNG2. These results were in complete agreement with co-immunoprecipitation experiments performed from 293T cells expressing HA-Vpr proteins in combination with GFP-UNG2 (Fig. 3D). Both wild-type and E17A HA-Vpr, but not VprW54R, were efficiently immunoprecipitated with GFP-UNG2 from co-transfected cell lysates. Similarly, we checked that the VprE17A mutant also retained the ability to associate with the DCAF1 subunit of the Cul4/DDB1 E3 ubiquitine ligase (Planelles and Benichou, 2009) that is critical for the G2-arrest activity induced by Vpr expression (data not shown).

Together, the data reported in Fig. 3 shows that the VprE17A variant is correctly incorporated into virus particles, localizes in the nucleus and efficiently interacts with its known UNG2 cellular partner required for modulation of the virus mutation rate.

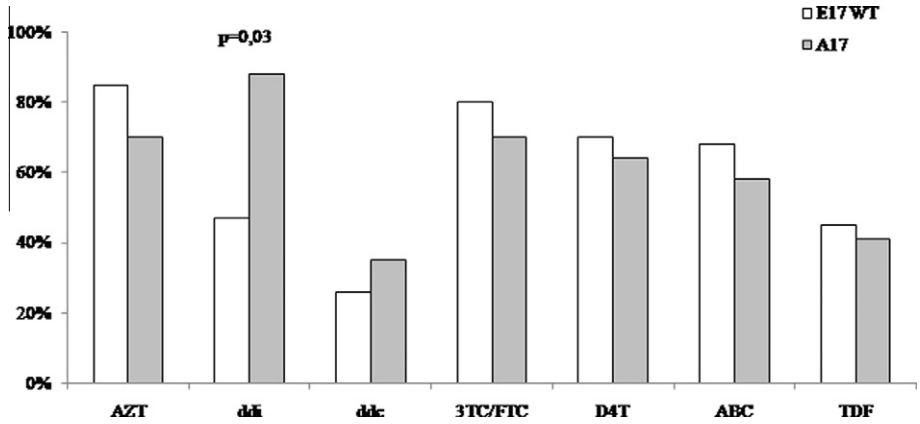

Fig. 2. Percentage of patients having received nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) in their history and harboring either E17 (WT) or A17. We observed a unique association between E17A and didanosine. Similar analyses were performed for other drug classes: non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), anti-integrase but failed to show any association (*Supplementary* file 1). ddI: didanosine, ddC: zalcitabine, 3TC: lamivudine, FTC: emtricitabine, D4T: stavudine, ABC: abacavir, TDF: ténofovir disoproxil fumarate.

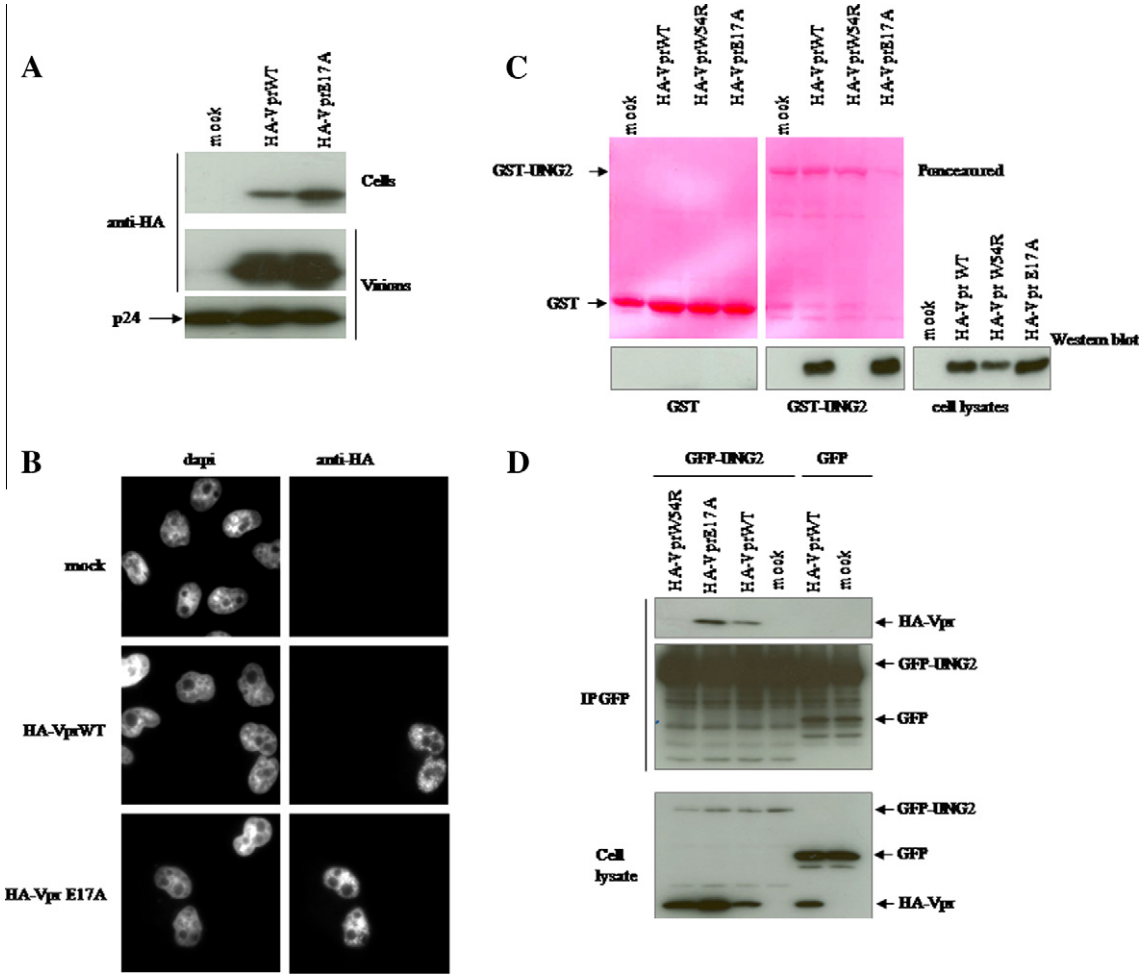

Fig. 3. Biochemical characterization and intracellular distribution of the Vpr E17A variant. (A) Packaging assay of the HA-tagged Vpr proteins into virus particles. Virions were produced from 293T cells co-transfected with an HIV-1-based packaging vector lacking the *vpr* gene in combination with the empty plasmid (mock) or with plasmids for expression of the wild type or mutated HA-tagged Vpr protein. 48 h later, proteins from cell and virion lysates were separated by SDS–PAGE and analyzed by Western blotting with anti-HA and anti-CAp24 antibodies. (B) Subcellular distribution of the HA-tagged Vpr proteins. HeLa cells were transfected with the empty plasmid (mock) or plasmids for expression of the wild type or mutated HA-Vpr protein and were then analyzed 48 h after transfection by immunofluorescence. Cells were fixed, permeabilized, and subsequently stained with anti-HA and DAPI (4′,6′-diamidino-2-phenylindole). Cells were analyzed by epifluorescence microscopy, and images were acquired by using a charge-coupled device camera. (C) *In vitro* pulldown assay for Vpr binding to UNG2. Lysates from 293T cells transfected with the empty plasmid (mock) or plasmids for expression of the wild type or mutated HA-Vpr protein (right panel) were incubated with equal amounts of either GST or GST-UNG2 (upper panels, Ponceau red) immobilized on GSH–Sepharose beads. Bound proteins were then analyzed by immunoblotting with anti-HA (lower panels). (D) Co-precipitation analysis of Vpr and UNG2 proteins. 293T cells transfected with the empty plasmid (mock) or plasmids for expression of the wild type or mutated HA-Vpr protein in combination with either GFP or GFP-UNG2 expression plasmids (lower panel, Cell lysate) were lyzed and subjected to immunoprecipitation with anti-GFP. Precipitates were then analyzed by immunoblotting with anti-HA (upper panel) and anti-GFP (middle panel).

3.4. Impact of the Vpr E17A variant on virus replication in a WT backbone and when combined with TAMs-1

To further investigate the potential role of E17A mutation, we studied its impact on virus replication in the context of the full-length virus. We introduced the Vpr E17A (E17A) mutation within the NL4-3 molecular clone and tested its replication kinetics in MT-2 cells. At day 5 and day 7 postinfection, levels of p24 production of E17A was 130% and 97% of the WT levels, respectively, showing that the E17A variant is at least as fit as NL4-3 WT (Fig. 4A). To gain a better understanding of the positive association of E17A with TAMs-1, we also generated NL4-3 site-directed mutants with M41L, L210W and T215Y in RT (TAMs-1) in combination or not with E17A (E17A + TAMs-1). Previous data have shown that the fitness of HIV-1 subtype B harboring TAMs-1 is reduced when compared to HIV-1 WT (Armstrong et al., 2009). Here, we asked if the E17A mutation would compensate for the reduced fitness of HIV-1 containing TAMs-1. As expected, HIV-1 TAMs-1 alone had reduced fitness in MT-2 cells (50% of WT levels at day 5 and 58% of WT levels at day 7 postinfection). When combined with E17A, HIV-1 E17A + TAMs-1 mutant showed similar replication capacity compared to viruses with TAMs-1 alone, indicating that E17A mutation does not compensate for the loss of fitness induced by TAMs-1 (Fig. 4A).

3.5. Phenotypic drug susceptibility of E17A HIV-1 in a WT backbone and when combined with TAMs-1

Because of the association of E17A with TAMs-1 (M41L, L210W and T215Y) within HIV-1 sequences from patients using didanosine, we next examined the impact of E17A alone or in combination with TAMs-1 on AZT and didanosine susceptibility by phenotypic assays (Fig. 4B, C). Phenotypic assays were performed three times. In didanosine susceptibility assays, TAMs-1 viruses showed no significant difference in didanosine susceptibility compared to WT NL4-3 (Fig. 4B). Similarly, the E17A mutant did not show reduced susceptibility to didanosine compared to WT NL4-3. However, when combined together, E17A + TAMs-1 demonstrated a 3.28-fold (range 2.9–3.6-fold) decrease in didanosine susceptibility compared to TAMs-1 alone (Fig. 4B). This level of resistance is close to that induced by a K65R mutant (used as a control) which demonstrated a 4-fold (range: 3–4.7-fold) decreased susceptibility to didanosine. Whereas E17A alone does not seem to confer resistance to didanosine, the data reported in Fig. 4B indicate that its association with TAMs-1 confers resistance to didanosine at a level close to the resistance induced by K65R. In AZT phenotypic assays, the E17A mutant showed no decreased susceptibility to AZT in comparison with NL4-3_{WT} (fold 1,2). When combined with TAMs-1 (E17A + TAMs-1), no further decreased susceptibility to AZT was observed as compared to TAMs-1 alone (Fig. 4C): indeed, TAMs-1 demonstrated about 80-fold decreased susceptibility to AZT (range: 65–97-fold) and E17A + TAMs-1 showed similar results (range: 75–95-fold change). Taken together, these data suggest that whereas Vpr E17A does not seem to confer resistance to AZT (whether associated to TAMs-1 or not), resistance to didanosine is observed when Vpr E17A is combined with TAMs-1 compared to viruses harboring TAMs-1 alone.

4. Discussion

Several studies have suggested a role for HIV-1 Vpr in modulating the viral mutation rate during the course of infection. Vpr has been found to incorporate the nuclear form uracil-DNA glycosylase 2 (UNG2) into HIV-1 virions. UNG-2 is a cellular DNA-repair enzyme involved in nucleotide-excision repair, and its incorporation in virions is correlated with the ability of Vpr to alter the mutation

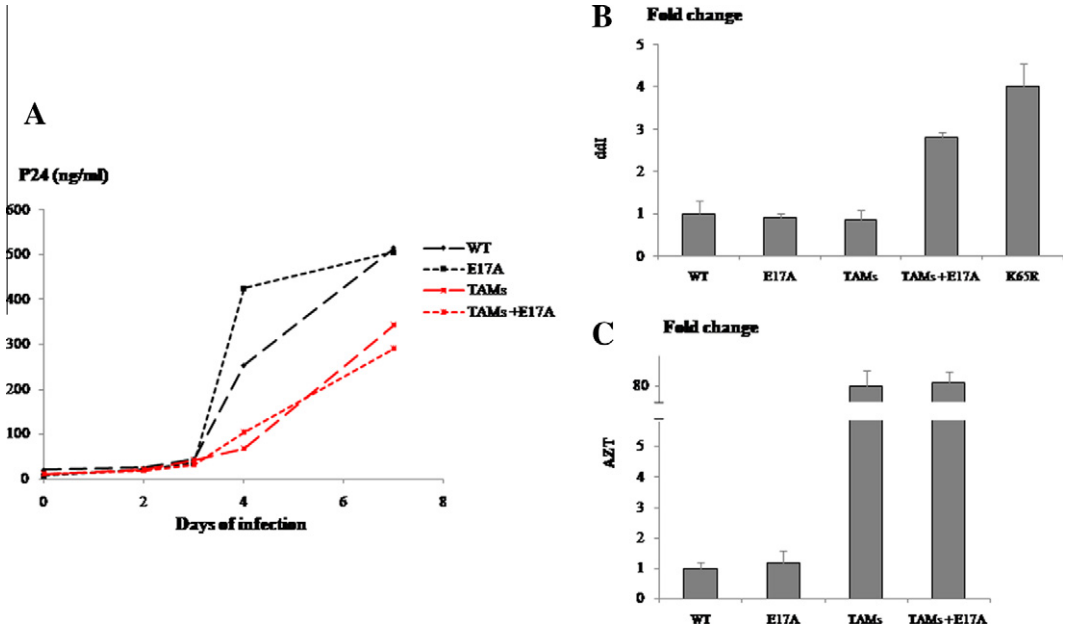

Fig. 4. Virological characterization of the VPR E17A. (A) Replication kinetics of RT and Vpr mutants. Replication kinetics of full-length NL4-3 WT and mutants derived from NL4-3: Vpr-E17A (E17A), RT-M41L, L210W, T215Y (TAMs) and Vpr-E17A + RT-M41L, L210W, T215Y (TAMs + E17A) viruses were determined using MT-2 T cells. Supernatants were collected every 1–3 days and assayed for viral p24 antigen. (B–C) Resistance of RT and Vpr mutants to didanosine. Hela-P4 Cells were infected, in triplicate, with NL4-3 WT (WT), and mutants derived from NL4-3: Vpr-E17A (E17A), RT-K65R (K65R), RT-[M41L, L210W, T215Y] (TAMs) and Vpr-E17A + RT-[M41L, L210W, T215Y] (E17A + TAMs) viruses (equivalent of 5 ng of p24gag antigen). The single cycle titers of viruses were determined 48 h after infection by quantifying β-galactosidase activity in HeLa-P4 lysates in a colorimetric assay (the CPRG assay). Cells were infected with viruses and grown in the presence of increasing concentrations of didanosine (B) or AZT (C). The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration giving 50% inhibition of β-galactosidase levels with respect to untreated infected cells. Drug susceptibility results were expressed as the fold change in susceptibility, defined as the ratio of the IC_{50} of the mutant variant to that of NL4-3 wild-type. Assays were performed three times.

rate of HIV-1 (Chen et al., 2004; Mansky, 1996). Additionally, a number of studies have confirmed that Vpr is contained within the reverse transcription complex (RTC) with the viral genome and some other viral proteins, including reverse transcriptase, integrase, nucleocapsid (NCp7), and small amounts of matrix (MA, p17Gag) (Farnet and Haseltine, 1991; Fassati and Goff, 2001; Le Rouzic and Benichou, 2005) in the early steps of the virus life cycle. Then, Vpr could remain associated with the viral DNA within 4–16 h after infection (Fassati and Goff, 2001).

Based on these observations, we speculated that Vpr could participate in viral diversity during the course of infection leading to the emergence of drug resistance associated mutations under antiretroviral treatment, either through the modulation of the virus mutation rate (Chen et al., 2004; Mansky, 1996) or, hypothetically, through direct or indirect interaction with RT within the RTC. We aimed at investigating Vpr alleles in patients failing ARV to assess whether their polymorphisms could have contributed in viral escape to ARV. We found a mutation in Vpr (E17A) located in the first α-helix to be associated with antiretroviral treatment failure. The first α-helix has been involved in several Vpr properties, including incorporation into virus particles and concentration of the protein in the nucleus and at the nuclear envelope (see Andersen et al. (2008), for review); nuclear localization of Vpr could be important for the Vpr requirement for efficient virus replication in non-dividing cells such as macrophages, but also for the Vpr-induced G2 arrest activity (de Noronha et al., 2001; Jacquot et al., 2007, 2009). Single-point Vpr mutants within this region showed significant alteration in these Vpr functions (see Andersen et al. (2008), for review). Here, we investigated whether Vpr E17A also alters the above-mentioned Vpr properties. However, our results indicate that this mutant has no impact on Vpr activity in terms of binding to its known cellular partners, subcellular localization and incorporation into virions. Specifically, E17A substitution had no impact on Vpr binding to UNG2, suggesting that this substitution has no impact on the viral mutation rate, thus excluding this mechanism as responsible for drug escape.

Considering drug resistance analysis in our HAART-treated population, Vpr E17A was associated with 3 thymidine analog mutations (TAMs) in RT (i.e., M41L, L210W and T215Y), but no association with RAMs to NNRTI or PIs was observed. By analyzing treatment histories of patients, we also observed a unique association between E17A and didanosine. Resistance to didanosine is frequently associated with mutations L74 V or K65R in RT (St Clair et al., 1991; Zhang et al., 1994) but has also been associated with viruses harboring a high numbers of TAMs (Flandre et al., 2007; Marcelin et al., 2005; Whitcomb et al., 2003). HIV-1 RT has the innate capacity to remove active metabolites of RT inhibitors such as didanosine in the presence of physiological concentration of ATP (Meyer et al., 1998). Thymidine analog mutations (TAMs) enhance NRTIs removal leading to drug resistance. *In vitro* assays using recombinant HIV-1 RT enzymes harboring TAMs compared levels of removal of NRTIs and demonstrated that AZT was the most efficiently removed NRTI and that the removal of didanosine was minimal (Naeger et al., 2002). This result is consistent with data indicating that in the context of a few number of TAMs, there is no or little impact on didanosine susceptibility (Flandre et al., 2007; Molina et al., 2005; Trivedi et al., 2008). In our study, using phenotypic assays, we confirmed that the presence of 3 TAMs-1 (M41L, L210W and T215Y) within the RT sequence does not significantly change didanosine susceptibility. However, we show that when combined with Vpr E17A, viruses with TAMs-1 demonstrate a significant reduction in susceptibility to didanosine. We also show that E17A does not enhance resistance to AZT (whether associated with TAMs or not); this observation reinforces the hypothesis that the association between TAMs and Vpr E17A in patients sequences would be more likely related to a potentializing effect of viruses with TAMs in resistance to didanosine rather than a potential role in AZT resistance. Whether Vpr E17A increases the excision process of didanosine induced by TAMs is still to be determined.

In conclusion, our results clearly show an association between Vpr E17A, TAMs (M41L, L210W, and T215Y) and the use of didanosine. Consistently with phenotypic drug susceptibility assays, the observed association between TAMs and Vpr E17A in patients' sequences is related to a potentializing effect of viruses with TAMs in enhancing resistance to didanosine. One possible explanation is that the use of didanosine in viruses harboring TAMs may select for mutations in Vpr that, in turn, enhance resistance to didanosine. Alternatively, viruses harboring Vpr E17A before treatment may be more likely to develop resistance to didanosine when this drug is introduced into a regimen and when the patient is infected with a virus containing TAMs. Longitudinal studies need to be conducted to elucidate the kinetic of appearence of these mutations. As for mechanistic respects, functional and structural analysis of interaction between Vpr and RT should better characterize this association, specifically to determine if Vpr plays a role in the excision process induced by TAMs. Similarly, because integrase is also involved in the reverse transcription complex and has been shown to interact with Vpr (Gleenberg et al., 2007) studies evaluating the role of Vpr in resistance to integrase inhibitors should be conducted.

Competing Interests

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.11.008.

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