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# Natural resistance of human immunodeficiency virus type 2 to zidovudine

Patrick Reid, Hamish MacInnes, Mian-er Cong, Walid Heneine, J. Gerardo García-Lerma*

*Laboratory Branch, Division of HIV/AIDS Prevention, National Center for HIV, STD, and TB Prevention, Centers for Disease Control and Prevention, Atlanta, GA 30329, USA*

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**Abstract**

Zidovudine (AZT) is a reverse transcriptase (RT) inhibitor widely used to treat persons infected with HIV-1 and HIV-2. Recent data on treated patients suggest differences in the antiviral activity of AZT between HIV-1 and HIV-2. We evaluated the antiviral activity of AZT on HIV-2 by using multiple approaches including in vitro selection experiments, analysis of growth kinetics with AZT, and phenotypic testing. A total of 5 wild-type (WT) HIV-2 viruses were used in the analysis. For comparison, 4 control WT HIV-1 strains and one HIV-1 mutant carrying the 215S mutation were evaluated in parallel. All 5 HIV-1 isolates acquired AZT resistance mutations after 3–6 passages with AZT or a 4- to 32-fold increase in AZT concentration. Among these viruses, the fastest selection of resistance was seen in HIV-$1_{S215}$, which acquired S215Y (1-nucleotide change only) at passage 3 after only 17 days in culture. In contrast, none of the 5 HIV-2 viruses that naturally have S215 acquired S215Y/F or any other RT mutation during 10 passages with AZT (1025-fold increase in AZT concentration). In the presence of AZT + didanosine (ddI), 3 of the 5 HIV-1 isolates acquired AZT or ddI resistance mutations, while only ddI resistance mutations were seen in HIV-2 (4 of 5 isolates). All HIV-2 viruses replicated efficiently in high AZT concentrations and were about 200-fold less sensitive to AZT than HIV-1. In contrast, HIV-2 and HIV-1 were equally susceptible to ddI, a finding consistent with the selection of HIV-2 mutants with AZT + ddI. Our results demonstrate that the activity of AZT on HIV-2 is lower than previously thought, and emphasize the need for novel antiretroviral drugs specific for HIV-2.
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*Keywords:* HIV-2; Zidovudine; Drug resistance

## Introduction

Human immunodeficiency virus type 2 (HIV-2) was first identified in 1985 in Senegal and is endemic in most West African countries (reviewed in Reeves and Doms, 2002). A large number of HIV-2 infections are also found in other geographical regions such as Western Europe and Asia (Reeves and Doms, 2002). HIV-2 infection differs clinically from HIV-1 infection by having a longer clinical latency, lower perinatal and sexual transmission rates, and less marked immunological abnormalities in asymptomatic individuals (Reeves and Doms, 2002). Like HIV-1, HIV-2 causes AIDS, and therefore, persons infected with HIV-2 may benefit from treatment with antiretroviral drugs including reverse transcriptase (RT) and protease inhibitors. However, information on how HIV-2-infected persons should be best treated is limited because of the absence of randomized clinical trials and because most infections occur in developing countries where few patients are treated. Drug selection for HIV-2 treatment has been generally guided by in vitro studies on the activity of available anti-HIV-1 drugs. Such studies have found comparable susceptibility to nucleoside RT inhibitors (NRTIs) and protease inhibitors among HIV-1 and HIV-2 with the possible exception of amprenavir, and documented natural resistance of HIV-2 to non-nucleoside RT inhibitors (NNRTIs) (Cox et al., 1994; Larder et al., 1990; Tantillo et al., 1994; Witvrouw et al., 1999, 2004).

AZT was the first antiretroviral drug approved for the treatment of HIV-1-infected persons and has been found to

* Corresponding author. Laboratory Branch, Centers for Disease Control and Prevention, 1600 Clifton Road NE, MS G-19, Atlanta, GA 30333, USA. Fax: +1 404 639 1174.
*E-mail address:* GGarcia-lerma@cdc.gov (J.G. García-Lerma)

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have a broad antiretroviral activity (García-Lerma et al., 2001b; Qari et al., 2001). Although data from HIV-2-infected patients treated with AZT and other NRTIs are limited to small cohorts of patients, several observations suggest differences in the antiviral activity of AZT between HIV-2 and HIV-1 (Adje-Toure et al., 2003; Brandin et al., 2003; Descamps et al., 2004; Rodes et al., 2000; van der Ende et al., 2000, 2003). The genotypic analysis of HIV-2 RT sequences from AZT-treated patients has shown a remarkably low frequency of selection of AZT mutations such as K70R or 215Y/F, which in HIV-1 are commonly associated with high levels of AZT resistance (Adje-Toure et al., 2003; Brandin et al., 2003; Descamps et al., 2004; Rodes et al., 2000; van der Ende et al., 2000, 2003). For instance, Rodes et al. (2000) found absence of 215Y/F in 11 HIV-2-infected patients that were treated for 7–68 months with AZT alone or in combination with other NRTIs, and only one patient acquired the K70R mutation. Similar findings were recently reported by Adje-Toure et al. (2003), who found 215Y in only one of 18 patients treated with AZT-containing regimens, and by van der Ende et al. (2003), who found absence of 215Y/F or K70R in 6 patients who failed AZT or d4T-containing regimens. In all these studies, Q151M was the primary mutation associated with the use of AZT and other NRTIs (Adje-Toure et al., 2003; Brandin et al., 2003; Descamps et al., 2004; Rodes et al., 2000; van der Ende et al., 2000, 2003), as opposed to HIV-1 in which 215Y/F represents the preferential pathway for NRTI resistance (Brun-Vezinet et al., 1997; Shafer et al., 1994, 1995). The striking differences in the frequency of AZT mutations and in mutational pathways of resistance between HIV-1 and HIV-2 have not been seen for other NRTIs such as lamivudine (3TC), which selects for the M184V mutation in both HIV-1 and HIV-2 (Adje-Toure et al., 2003; Brandin et al., 2003; Descamps et al., 2004; Rodes et al., 2000; van der Ende et al., 2000, 2003).

The low frequency of selection of 215Y/F seen in HIV-2-infected persons treated with AZT and other NRTIs is surprising since WT HIV-2 RT sequences have Ser at position 215 (TCT), which differs from the mutant 215Y/F (TAT and TTT, respectively) by a single nucleotide change as opposed to the 2 nucleotide changes required for the WT T215 of HIV-1. Therefore, it was expected that selection of 215Y/F would be favored in HIV-2 since HIV-1 mutants that differ from 215Y/F by a single nucleotide rapidly acquire 215Y with AZT both in vitro and in vivo (de Ronde et al., 2000; García-Lerma et al., 2001a; Violin et al., 2004). These findings suggest that the S215Y or F mutations might confer a minimal selective advantage for HIV-2 because of a reduced fitness or because they confer minimal resistance to AZT. Alternatively, it is possible that differences in AZT selective pressures might be responsible for the low frequency of selection of AZT mutations in HIV-2. Therefore, additional analysis of the activity of AZT on HIV-2 is necessary to fully understand the differences in patterns and frequency of AZT mutations seen between HIV-1 and HIV-2-infected persons.

In the present study, we have re-evaluated the antiviral activity of AZT on HIV-2 by using multiple approaches including in vitro selection experiments, analysis of growth kinetics in the presence of AZT, and drug susceptibility testing. We demonstrate that the susceptibility of HIV-2 to AZT is lower than previously thought.

## Results

### *Absence of selection of AZT-resistant HIV-2 during sequential passages with AZT*

We first investigated patterns of mutations (amino acids 32–224) in HIV-2 isolates (CDC313019, CDC77618, GB122HU, CBL20/H9, and ROD) during sequential passages with increasing concentrations of AZT. For comparison, the kinetics of selection of resistance mutations were also evaluated in 5 control HIV-1 isolates including 4 wild type (WT) strains (HXB2, IIIB/H9, CC/H9, and LAI) and one site-directed mutant carrying the T215S mutation ($HXB2_{S215}$).

In the presence of AZT, all 5 HIV-1 strains acquired AZT resistance mutations after 3–6 passages or an increase in the concentration of AZT of 4- to 32-fold (from 0.024 to 0.096 or 0.77 μg/ml, respectively) (Table 1). Among these viruses, the fastest selection of resistance was seen in $HXB2_{S215}$, which acquired the S215Y mutation at passage 3 after only 17 days in culture. Of the remaining 4 HIV-1 strains, HXB2 acquired K70R at passage 5 (day 36) followed by D67N at passage 8 (day 77), IIIB/H9 acquired K70R at passage 6 (day 41) followed by a mixture of T215S, I, and F at passage 7 (day 48), CC/H9 acquired D67N at passage 5 (41 days) followed by K70R at passage 6 (day 51), and LAI acquired D67N at passage 6 (day 38) followed by K70R at passage 7 (day 50). In contrast to HIV-1, none of the 5 HIV-2 viruses acquired resistance mutations during 10 passages or an increase in the concentration of AZT of 1025-fold (from 0.024 to 24.6 μg/ml). Isolates GB122HU and CDC77618 acquired a 104K and a 64K mutation, respectively. However, 104K and 64K were also seen at baseline in CBL-20/H9, CDC77618, CDC310319, or ROD (see below), indicating that they represent natural polymorphisms.

To investigate if other mutations might have been selected outside amino acids 32–224, we expanded our sequence analysis of HIV-2 RT to amino acid 550 in isolates collected at passage 8 and 10 with AZT. RT sequences were then compared to the sequences obtained in baseline isolates. The results showed a mixture of 298L/P and a change from 387K/R to 387R in isolate CBL-20/H9 at passages 8 and 10, and a mixture of 466K/R in isolate CDC77618 at passages 8 and 10. Isolates GB122HU, CDC310319, and ROD showed absence of any RT mutation at these high AZT concentrations (not shown). Overall, these findings indicate little or no selective pressure by AZT during this range of drug concentrations. Concentrations of

Table 1
RT mutations in HIV-1 and HIV-2 during passages with AZT

| | Passage | AZT (μg/ml) | Time (days) | Cumulative time (days) | RT mutations |
|---|---|---|---|---|---|
| HIV-2 | | | | | |
| CDC310319 | 1 | 0.024 | 4 | 4 | n.d. |
| | 2 | 0.048 | 4 | 8 | n.d. |
| | 3 | 0.096 | 5 | 13 | – |
| | 4 | 0.19 | 4 | 17 | n.d. |
| | 5 | 0.38 | 4 | 21 | n.d. |
| | 6 | 0.77 | 4 | 25 | n.d. |
| | 7 | 1.54 | 4 | 29 | n.d. |
| | 8 | 6.14 | 5 | 34 | – |
| | 9 | 12.3 | 6 | 40 | n.d. |
| | 10 | 24.6 | 5 | 45 | – |
| CDC77618 | 1 | 0.024 | 4 | 4 | n.d. |
| | 2 | 0.048 | 3 | 7 | n.d. |
| | 3 | 0.096 | 5 | 12 | n.d. |
| | 4 | 0.19 | 6 | 18 | n.d. |
| | 5 | 0.38 | 6 | 24 | n.d. |
| | 6 | 0.77 | 5 | 29 | n.d. |
| | 7 | 1.54 | 4 | 33 | n.d. |
| | 8 | 6.14 | 5 | 38 | – |
| | 9 | 12.3 | 6 | 44 | – |
| | 10 | 24.6 | 5 | 49 | R64R/K[a] |
| CBL-20/H9 | 1 | 0.024 | 4 | 4 | n.d. |
| | 2 | 0.048 | 3 | 7 | n.d. |
| | 3 | 0.096 | 4 | 11 | n.d. |
| | 4 | 0.19 | 4 | 15 | – |
| | 5 | 0.38 | 6 | 21 | n.d. |
| | 6 | 0.77 | 5 | 26 | n.d. |
| | 7 | 1.54 | 4 | 30 | n.d. |
| | 8 | 6.14 | 5 | 35 | – |
| | 9 | 12.3 | 6 | 41 | n.d. |
| | 10 | 24.6 | 5 | 46 | – |
| GB122 HU | 1 | 0.024 | 4 | 4 | n.d. |
| | 2 | 0.048 | 4 | 8 | n.d. |
| | 3 | 0.096 | 4 | 12 | – |
| | 4 | 0.19 | 4 | 16 | R104R/K |
| | 5 | 0.38 | 4 | 20 | R104R/K |
| | 6 | 0.77 | 4 | 24 | R104R/K |
| | 7 | 1.54 | 4 | 28 | R104R/K |
| | 8 | 6.14 | 4 | 32 | R104R/K |
| | 9 | 12.3 | 5 | 37 | n.d. |
| | 10 | 24.6 | 5 | 42 | R104R/K |
| ROD | 1 | 0.024 | 4 | 4 | n.d. |
| | 2 | 0.048 | 4 | 8 | n.d. |
| | 3 | 0.096 | 4 | 12 | – |
| | 4 | 0.19 | 4 | 16 | – |
| | 5 | 0.38 | 6 | 22 | n.d. |
| | 6 | 0.77 | 5 | 27 | n.d. |
| | 7 | 1.54 | 4 | 31 | n.d. |
| | 8 | 6.14 | 5 | 36 | – |
| | 9 | 12.3 | 5 | 41 | n.d. |
| | 10 | 24.6 | 5 | 46 | – |
| HIV-1 | | | | | |
| $HXB2_{S215}$ | 1 | 0.024 | 7 | 7 | n.d. |
| | 2 | 0.048 | 4 | 11 | – |
| | 3 | 0.096 | 6 | 17 | S215Y |
| | 4 | 0.19 | 6 | 23 | S215Y |
| | 5 | 0.38 | 6 | 29 | n.d. |
| | 6 | 0.77 | 6 | 35 | S215Y, H221H/Y |
| | 7 | 1.54 | 6 | 41 | n.d. |
| | 8 | 6.14 | 11 | 52 | S215F/Y, H221H/Y |
| HXB2 | 1 | 0.024 | 7 | 7 | n.d. |
| | 2 | 0.048 | 5 | 12 | n.d. |
| | 3 | 0.096 | 6 | 18 | n.d. |
| | 4 | 0.19 | 8 | 26 | – |
| | 5 | 0.38 | 10 | 36 | K70K/R |
| | 6 | 0.77 | 8 | 44 | K70R |
| | 7 | 1.54 | 12 | 60 | n.d. |
| | 8 | 6.14 | 17 | 77 | D67N, K70R |
| IIIB-H9 | 1 | 0.024 | 4 | 4 | n.d. |
| | 2 | 0.048 | 5 | 9 | n.d. |
| | 3 | 0.096 | 6 | 15 | n.d. |
| | 4 | 0.19 | 8 | 23 | – |
| | 5 | 0.38 | 9 | 32 | – |
| | 6 | 0.77 | 8 | 41 | K70R |
| | 7 | 1.54 | 7 | 48 | K70R, T215S/I/F |
| | 8 | 6.14 | 10 | 58 | L214F, T215F, K70K/R |
| CC/H9 | 1 | 0.024 | 4 | 4 | n.d. |
| | 2 | 0.048 | 8 | 12 | n.d. |
| | 3 | 0.096 | 6 | 18 | n.d. |
| | 4 | 0.19 | 8 | 26 | – |
| | 5 | 0.38 | 15 | 41 | D67D/N |
| | 6 | 0.77 | 10 | 51 | D67N/D, K70R/K |
| | 7 | 1.54 | 9 | 60 | D67N, K70R |
| | 8 | 6.14 | 10 | 70 | D67N, K70R |
| LAI | 1 | 0.024 | 4 | 4 | n.d. |
| | 2 | 0.048 | 5 | 9 | n.d. |
| | 3 | 0.096 | 6 | 15 | n.d. |
| | 4 | 0.19 | 6 | 21 | – |
| | 5 | 0.38 | 7 | 28 | – |
| | 6 | 0.77 | 10 | 38 | D67D/N |
| | 7 | 1.54 | 12 | 50 | D67N, K70R/K |
| | 8 | 6.14 | 10 | 60 | D67N, K70R |

–, no mutations observed; n.d., not done.
[a] Mixed genotype. The first amino acid represents the predominant genotype observed in the mixture.

AZT higher than 24.6 μg/ml resulted in a significant toxicity in the culture and could not be evaluated (not shown).

### *Selection of HIV-2 mutants by sequential passages with AZT and ddI*

We next compared RT mutations selected in HIV-1 and HIV-2 during culture with both AZT and ddI. Table 2 shows that 3 of the 5 HIV-1 strains acquired AZT or ddI resistance mutations such as S215F, D67N, and K65R after 4 to 6 passages with AZT + ddI. Similarly, 4 of the 5 HIV-2 viruses acquired mutations that are known to be associated with ddI resistance in HIV-1. Of these viruses, CDC77618 and ROD acquired the K65R mutation and CBL-20/H9 and

Table 2
RT mutations in HIV-1 and HIV-2 during passages with AZT and ddI

| | Passage | AZT/ddI (μg/ml) | Time (days) | Cumulative time (days) | RT mutations |
|---|---|---|---|---|---|
| HIV-2 | | | | | |
| CDC310319 | 1 | 0.003/2 | 7 | 7 | – |
| | 2 | 0.006/4 | 7 | 14 | – |
| | 3 | 0.012/8 | 6 | 20 | – |
| | 4 | 0.024/16 | 8 | 28 | – |
| | 5 | 0.048/32 | 9 | 37 | – |
| | 6 | 0.096/64 | 16 | 53 | n.d. |
| | 7 | 0.19/128 | 12 | 65 | – |
| | 8 | 0.38/256 | 22 | 87 | – |
| CDC77618 | 1 | 0.003/2 | 6 | 6 | – |
| | 2 | 0.006/4 | 7 | 13 | – |
| | 3 | 0.012/8 | 9 | 22 | – |
| | 4 | 0.024/16 | 8 | 30 | – |
| | 5 | 0.048/32 | 9 | 39 | – |
| | 6 | 0.096/64 | 17 | 56 | K65K/R[a] |
| | 7 | 0.19/128 | 14 | 70 | K65R |
| | 8 | 0.38/256 | 22 | 92 | K65R, I189I/M |
| CBL-20/H9 | 1 | 0.003/2 | 7 | 7 | – |
| | 2 | 0.006/4 | 6 | 13 | – |
| | 3 | 0.012/8 | 7 | 20 | – |
| | 4 | 0.024/16 | 8 | 28 | – |
| | 5 | 0.048/32 | 9 | 37 | – |
| | 6 | 0.096/64 | 15 | 52 | n.d. |
| | 7 | 0.19/128 | 12 | 64 | M184I |
| GB122 HU | 1 | 0.003/2 | 7 | 7 | – |
| | 2 | 0.006/4 | 5 | 12 | – |
| | 3 | 0.012/8 | 7 | 19 | – |
| | 4 | 0.024/16 | 8 | 27 | – |
| | 5 | 0.048/32 | 7 | 34 | – |
| | 6 | 0.096/64 | 17 | 51 | M184I |
| | 7 | 0.19/128 | 12 | 63 | M184I, R104K/R |
| | 8 | 0.38/256 | 22 | 85 | M184I, R104K |
| ROD | 1 | 0.003/2 | 5 | 5 | – |
| | 2 | 0.006/4 | 5 | 10 | – |
| | 3 | 0.012/8 | 7 | 17 | – |
| | 4 | 0.024/16 | 8 | 25 | – |
| | 5 | 0.048/32 | 9 | 34 | – |
| | 6 | 0.096/64 | 17 | 51 | – |
| | 7 | 0.19/128 | 14 | 65 | – |
| | 8 | 0.38/256 | 22 | 87 | K64R, K65R |
| HIV-1 | | | | | |
| $HXB2_{S215}$ | 1 | 0.003/2 | 8 | 8 | – |
| | 2 | 0.006/4 | 12 | 20 | – |
| | 3 | 0.012/8 | 20 | 40 | n.d. |
| | 4 | 0.024/16 | 31 | 71 | H221Y |
| | 4b | 0.024/16 | 32 | 103 | H221Y, S215F |
| HXB2 | 1 | 0.003/2 | 7 | 7 | – |
| | 2 | 0.006/4 | 12 | 19 | – |
| | 3 | 0.012/8 | 15 | 34 | n.d. |
| | 4 | 0.024/16 | 29 | 63 | E138K |
| | 4b | 0.024/16 | 39 | 102 | n.d. |
| | 4c | 0.024/16 | 20 | 122 | E138K |
| | 5 | 0.048/32 | 33 | 155 | E138K |
| B-H9 | 1 | 0.003/2 | 6 | 6 | – |
| | 2 | 0.006/4 | 8 | 14 | – |
| | 3 | 0.012/8 | 11 | 25 | – |
| | 4 | 0.024/16 | 21 | 46 | K65R |
| | 4b | 0.024/16 | 14 | 60 | K65R |
| | 5 | 0.048/32 | 17 | 77 | K65R |
| CC/H9 | 1 | 0.003/2 | 6 | 6 | – |
| | 2 | 0.006/4 | 7 | 13 | – |
| | 3 | 0.012/8 | 19 | 32 | n.d. |
| | 4 | 0.024/16 | 29 | 61 | – |
| | 4b | 0.024/16 | 39 | 100 | – |
| | 4c | 0.024/16 | 16 | 116 | – |
| | 5 | 0.048/32 | 52 | 168 | – |
| LAI | 1 | 0.003/2 | 6 | 6 | – |
| | 2 | 0.006/4 | 7 | 13 | – |
| | 3 | 0.012/8 | 11 | 24 | n.d. |
| | 4 | 0.024/16 | 21 | 45 | – |
| | 4b | 0.024/16 | 22 | 67 | – |
| | 4c | 0.024/16 | 12 | 79 | D67D/N, H221Y |
| | 5 | 0.048/32 | 17 | 96 | D67N, H221Y |

–, no mutations observed; n.d., not done.
[a] Mixed genotype. The first amino acid represents the predominant genotype observed in the mixture.

GB122HU acquired the M184I mutation. The absence of selection of HIV-2 mutants with AZT alone suggests that ddI may be driving the selection of K65R and M184I in HIV-2 during culture with AZT and ddI.

### *Selective pressure by AZT and AZT + ddI in HIV-1 and HIV-2*

The analysis of the relationship between the time required to observe full cytopathic effect (CPE) in culture and the increase in the concentration of drug during the passages may provide an indication of the selective pressure conferred by the drug/s. Fig. 1 illustrates such a relationship in both HIV-1 and HIV-2 passaged with AZT or AZT + ddI. In experiments done with WT HIV-1 and AZT, the time required to observe full CPE in the passages increased exponentially with the increase in AZT concentrations during passages, likely reflecting the selective pressure conferred by AZT and, possibly, an impairment of fitness associated with the selection of AZT resistance mutations. Similar exponential relationship was seen in both HIV-1 and HIV-2 during passages in the presence of AZT + ddI, supporting a drug selective pressure by AZT+ddI in these viruses. In contrast, the time required to observe full CPE in cultures done with WT HIV-2 and AZT was short and constant during all the passages (4.6 ± 0.07 days). Such a linear relationship was indicative of a minimal drug selective pressure in the wide range of AZT concentrations tested, a finding that is consistent with the absence of selection of AZT mutations. Fig. 1 also shows that the fluctuations in the levels of p24 or p27 antigen seen during the entire selection experiments were similar in HIV-1 and HIV-2, indicating

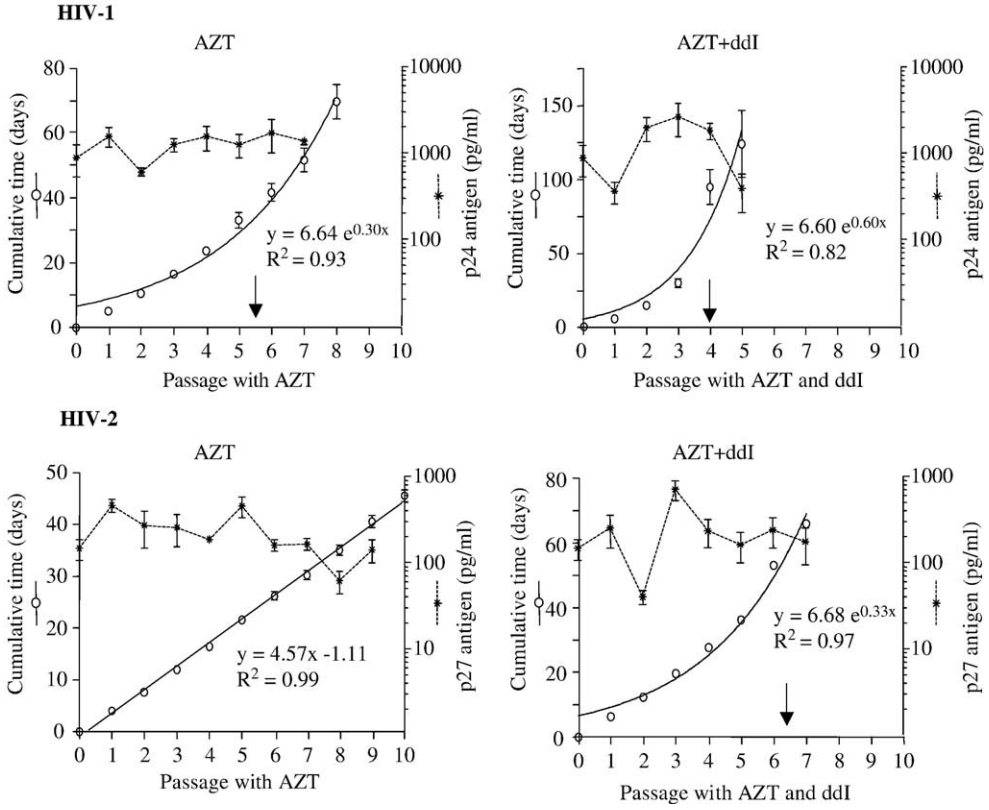


Fig. 1. Cumulative time in culture and levels of p24 or p27 antigen during passages with AZT or AZT + ddI in HIV-1 and HIV-2. The relationship between the passages with AZT or AZT + ddI, the cumulative time in culture, and the level of p24 or p27 antigen observed at the end of each passage are shown. Data reflect the mean cumulative time or p24/p27 antigen levels seen in all the HIV-1 or HIV-2 isolates (bars denote the standard error of the mean (SEM)). Arrows indicate the median passage at which an AZT or a ddI resistance mutation was seen.

that the observed differences in selective pressures were not associated with differences in virus production.

### *HIV-2 can replicate efficiently in the presence of a high concentration of AZT*

We next investigated the ability of HIV-2 to replicate in MT-4 cells in the presence of a high concentration of AZT. We selected a concentration of AZT of 12.3 μg/ml, which is 2800-fold higher than the median $EC_{50}$ value of AZT for HIV-1. Replication capacities were measured in baseline HIV-2 isolates, in HIV-2 isolates collected after 8 passages with AZT (Table 1, p8 isolates), and in baseline HIV-2 isolates that were adapted to grow in MT-4 cells by limited passages (5 passages) in the absence of drug. The replication kinetics of baseline HIV-1 and of HIV-1 isolates collected at passage 6 with AZT (Table 1, p6 isolates) were also evaluated in parallel cultures done with 1.6 μg/ml of AZT. Fig. 2 shows the kinetics of p27 or p24 antigen production seen in all these cultures.

All the 5 HIV-2 p8 isolates replicated efficiently in the presence of 12.3 μg/ml of AZT and showed detectable p27 antigen after 4 days of culture (Fig. 2A). Similarly, all the 5 baseline HIV-2 isolates replicated efficiently with AZT (Fig. 2B). However, the kinetics of p27 antigen production seen in these isolates were delayed compared to those of p8 isolates. For instance, isolates CDC310319, CDC77618, CBL-20/H9, and GB122HU showed little or no p27 antigen production during the first 10 days of culture followed by a rapid increase in p27 from day 10 to day 14. Fig. 2C shows that such a delay in virus production was minimal when the baseline isolates were previously adapted to grow in MT-4 cells, indicating that the observed differences in virus replication likely reflect a reduced ability of baseline isolates to replicate in MT-4 cells. A comparison of RT sequences between baseline HIV-2 isolates and isolates collected after 12–14 days of culture with AZT showed absence of RT mutations, indicating that the efficient replication seen with AZT was not due to the selection of AZT-resistant mutants (data not shown).

Fig. 2D shows the kinetics of HIV-1 replication in MT-4 cells in the presence of AZT. Three of the four baseline HIV-1 viruses tested ($HXB2_{S215}$, HXB2, and CC/H9) did not replicate with AZT, and only IIIB/H9 had evidence of low levels of viral replication at day 14. In contrast, the AZT-resistant HIV-1 isolates $HXB2_{S215Y}$ and $IIIB/H9_{K70R}$ that were selected at passage 6 with AZT were able to replicate efficiently in the presence of AZT, a finding confirming the reduced susceptibility of these mutants to AZT (Fig. 2D).

The ability of HIV-2 to replicate in high AZT concentrations was also evaluated in PBMCs and $CEM_{174}$ cells

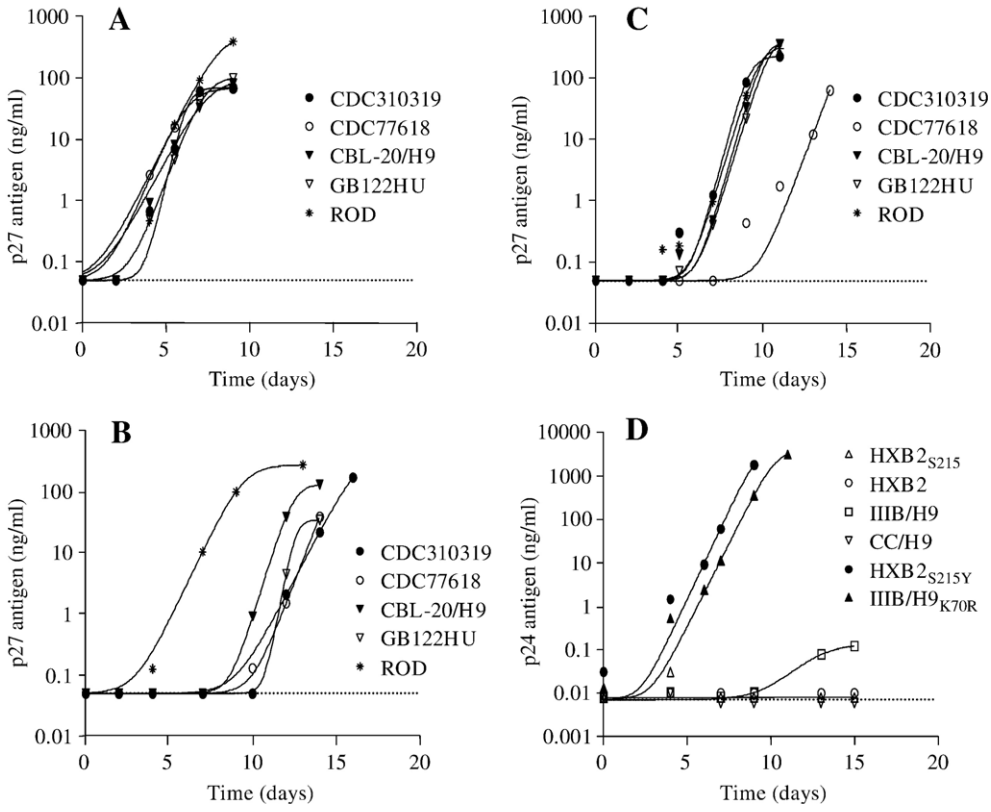


Fig. 2. Replication kinetics of HIV-2 and HIV-1 in the presence of 12.2 μg/ml or 1.6 μg/ml of AZT, respectively. (A) HIV-2 isolates collected after 8 passages with AZT (p8 isolates). (B) Baseline HIV-2 isolates. (C) Baseline HIV-2 isolates adapted to grow in MT-4 cells by limited passages in the absence of drug. (D) Baseline HIV-1 isolates ($HXB2_{S215}$, HXB2, IIIB/H9, and CC/H9) and HIV-1 isolates collected after 6 passages with AZT ($HXB2_{S215Y}$ and $IIIB/H9_{K70R}$; p6 isolates). Replication kinetics were evaluated in at least two independent experiments. A representative experiment is shown. A broken line denotes the limit of detection of the p24 and p27 assay.

(Fig. 3). In the absence of AZT, all the HIV-1 and HIV-2 isolates replicated efficiently in both PBMCs and $CEM_{174}$ cells (data not shown). In the presence of 2 μg/ml of AZT, all the 5 HIV-2 isolates replicated efficiently while little or no virus replication was seen in the 4 control WT HIV-1 isolates (Fig. 3). These findings indicate that the efficient replication of HIV-2 with AZT is not restricted to MT-4 cells, and confirm that HIV-2 is resistant to AZT.

*Susceptibility of HIV-2 to AZT and ddI and comparison with HIV-1*

We next compared the susceptibility to AZT and ddI among HIV-1 and HIV-2. We calculated $EC_{50}$ and $EC_{90}$ values for AZT and ddI using the MT-4/MTT assay in a regular and an extended format. In the regular format of the assay, $EC_{50}$ and $EC_{90}$ values were calculated after 5 days of culture. In the extended format, $EC_{50}$ and $EC_{90}$ values were calculated after 10 or 14 days of culture. Tables 3 and 4 show the $EC_{50}$ and $EC_{90}$ values for AZT and ddI seen at days 5, 10, and 14 in 4 baseline HIV-2 (CDC77618, CBL-20/H9, ROD, and GB122HU) and 4 baseline HIV-1 viruses (HXB2, $HXB2_{S215}$, CC/H9, and IIIB/H9).

At day 5, the median $EC_{50}$ value for AZT in HIV-2 was 0.010 μg/ml (range = 0.004–0.038) and was only 2.2-fold higher than the median $EC_{50}$ value seen in HIV-1 (0.0043 μg/ml, range = 0.0029–0.0058). However, at day 10, the median $EC_{50}$ value for AZT in HIV-2 (6.3 μg/ml; range = 0.29 to >25) was >140-fold higher than the median $EC_{50}$ value seen in HIV-1 (0.045 μg/ml; range = 0.018–0.084). Table 3 shows that while $EC_{50}$ values in HIV-1 only increased 10-fold from day 5 to day 10, such increase was >630-fold in HIV-2. At day 14, $EC_{50}$ values for AZT further increased in HIV-2 (median = 25 μg/ml; range = 11.9 to >25), while only a 2.8-fold change was seen in HIV-1 from day 10 to day 14.

Similar findings were seen when the $EC_{90}$ values were compared (Table 3). However, these values were already higher in HIV-2 than in HIV-1 at day 5. Table 3 shows that the median $EC_{90}$ value for AZT seen in HIV-2 at day 5 (0.47 μg/ml; range = 0.02–1.82) was 22.7-fold higher than the median $EC_{90}$ value seen in HIV-1 (0.021 μg/ml; range = 0.007–0.029).

The differences in AZT susceptibility between HIV-1 and HIV-2 were not due to an insufficient viral replication and lower cytotoxicity of HIV-2 compared to HIV-1. Fig. 4 shows the loss of MTT signal (OD at 540 nm) due to HIV-1 and HIV-2 replication seen in the absence of AZT and in the presence of a high AZT concentration (5 μg/ml). In the absence of AZT, both HIV-1 and HIV-2 showed maximal

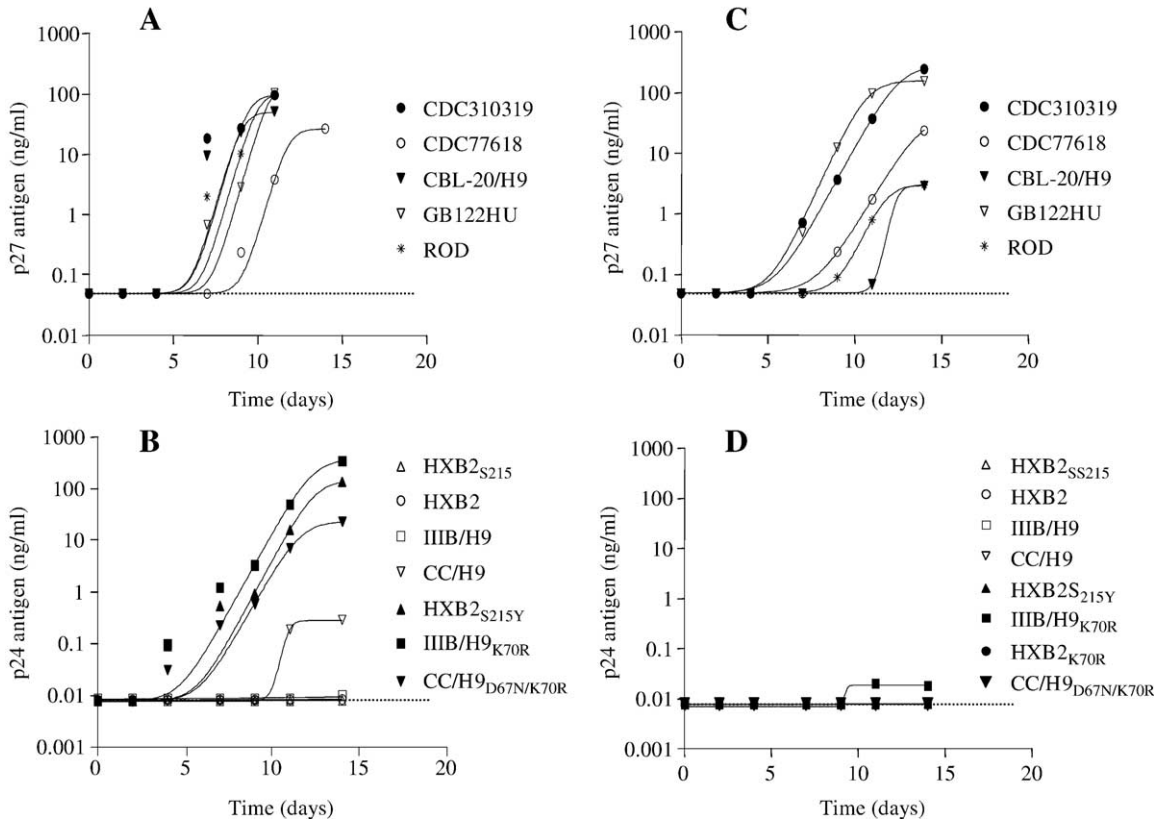


Fig. 3. Replication capacity of baseline HIV-1 and HIV-2 isolates in $CEM_{174}$ and PBMCs in the presence of 2 μg/ml AZT. (A) Replication of HIV-2 in $CEM_{174}$. (B) Replication of HIV-1 in $CEM_{174}$. (C) Replication of HIV-2 in PBMCs. (B) Replication of HIV-1 in PBMCs. Replication capacities were evaluated in two independent experiments. Mean p24 values from a representative experiment performed in duplicate ($CEM_{174}$) or quadruplicate (PBMCs) are shown. A broken line indicates the limit of detection of the p24 and p27 assay.

cytotoxicity at days 5, 10, and 14 as indicated by the low OD values seen in the MTT assay. In contrast, viral cytotoxicity at high AZT concentrations was low in both HIV-1 and HIV-2 at day 5, and increased overtime only in HIV-2, illustrating the lower protective effect of AZT on HIV-2 compared to HIV-1 (Fig. 4). Taken together, these findings demonstrate a lower antiviral activity of AZT on HIV-2, which may explain the absence of mutations during passages with AZT and the efficient replication seen at high AZT concentrations.

We also compared the susceptibility to ddI among HIV-1 and HIV-2 (Table 4). In contrast to AZT, the $EC_{50}$ values for

Table 3
Comparison of the $EC_{50}$ and $EC_{90}$ values for AZT between HIV-1 and HIV-2

| | $EC_{50}$ (μg/ml) | | | $EC_{90}$ (μg/ml) | | |
|---|---|---|---|---|---|---|
| | Day 5 | Day 10 | Day 14 | Day 5 | Day 10 | Day 14 |
| HIV-1 | | | | | | |
| HXB2 | 0.0035 | 0.018 | 0.019 | 0.023 | 0.03 | 0.03 |
| $HXB2_{S215}$ | 0.0029 | 0.019 | 0.082 | 0.007 | 0.31 | 0.18 |
| IIIB/H9 | 0.0050 | 0.084 | 0.375 | 0.029 | 0.17 | 0.82 |
| CC/H9 | 0.0058 | 0.071 | 0.165 | 0.018 | 0.21 | 0.70 |
| Median | 0.0043 | 0.045 | 0.124 | 0.021 | 0.19 | 0.44 |
| HIV-2 | | | | | | |
| ROD | 0.038 | >25[a] | >25 | 1.82 | >25 | >25 |
| CBL-20/H9 | 0.008 | 11.9 | >25 | 0.68 | >25 | >25 |
| CDC77618 | 0.011 | 0.72 | 11.9 | 0.25 | 3.28 | >25 |
| GB122HU | 0.004 | 0.29 | >25 | 0.02 | 0.78 | >25 |
| Median | 0.010 | 6.3 | 25 | 0.47 | >14 | >25 |
| Ratio HIV-2/HIV-1 | 2.2 | >140 | >202 | 22.7 | >74 | >57 |

[a] $EC_{50}$ values >25 μg/ml were given an arbitrary value of 25 for the calculation of medians.

Table 4
Comparison of the $EC_{50}$ and $EC_{90}$ values for ddI between HIV-1 and HIV-2

| | $EC_{50}$ (μg/ml) | | | $EC_{90}$ (μg/ml) | | |
|---|---|---|---|---|---|---|
| | Day 5 | Day 10 | Day 14 | Day 5 | Day 10 | Day 14 |
| HIV-1 | | | | | | |
| HXB2 | 0.55 | 3.57 | 3.34 | 2.7 | 6.8 | 7.2 |
| $HXB2_{S215}$ | 1.87 | 4.21 | 17.6 | 7.4 | 18 | 38 |
| IIIB/H9 | 2.25 | 12 | 15.4 | 7.3 | 31 | 36 |
| CC/H9 | 0.47 | 6.69 | 17.6 | 5.6 | 28 | 37 |
| Median | 1.21 | 5.45 | 16.5 | 6.45 | 23 | 36 |
| HIV-2 | | | | | | |
| ROD | 3.66 | 17.7 | 34.2 | 7.7 | 34 | 141 |
| CBL-20/H9 | 3.29 | 17.7 | 16.7 | 6.7 | 34 | 34 |
| CDC77618 | 3.28 | 9.3 | 15.8 | 7.4 | 30 | 33 |
| GB122HU | 0.72 | 5.1 | 18.2 | 2.4 | 26 | 38 |
| Median | 3.29 | 13.5 | 17.5 | 7.1 | 32 | 36 |
| Ratio HIV-2/HIV-1 | 2.7 | 2.5 | 1.1 | 1.1 | 1.4 | 1 |

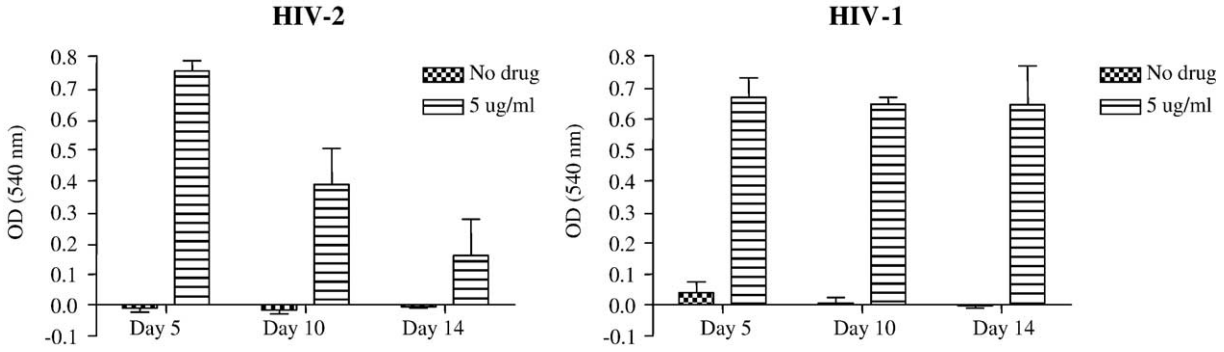


Fig. 4. Cytotoxicity of HIV-1 and HIV-2 isolates by the standard (day 5) and the modified (days 10 and 14) MT4/MTT phenotypic assay. Data show the mean cytotoxicity (OD at 540 nm) observed in the absence and in the presence of AZT (5 μg/ml) in 4 HIV-1 (HXB2, $HXB2_{S215}$, CC/H9, and IIIB/H9) and 4 HIV-2 (CDC77618, CBL20/H9, GB122HU, and ROD) isolates. Bars denote the standard error of the mean (SEM).

ddI were similar in HIV-1 and HIV-2 at days 5, 10, and 14. At day 5, the median $EC_{50}$ value in HIV-2 was 3.29 μg/ml (range = 0.72–3.66) and was only 2.7-fold higher than the median value calculated in HIV-1 (1.21 μg/ml; range = 0.47–2.25). At days 10 and 14, these values were 2.5- and 1.1-fold higher in HIV-2 than in HIV-1, respectively. Similar findings were seen when the $EC_{90}$ values were compared, further supporting that both viruses are equally susceptible to ddI. Fig. 5 illustrates the inhibition curves for AZT and ddI observed in all these viruses at days 5, 10, and 14.

*Enzymatic susceptibility of HIV-2 RT to AZT-TP*

To investigate if the observed AZT resistance of HIV-2 could be due to an increased ability of HIV-2 RT to discriminate against AZT-TP, we compared the $EC_{50}$ values

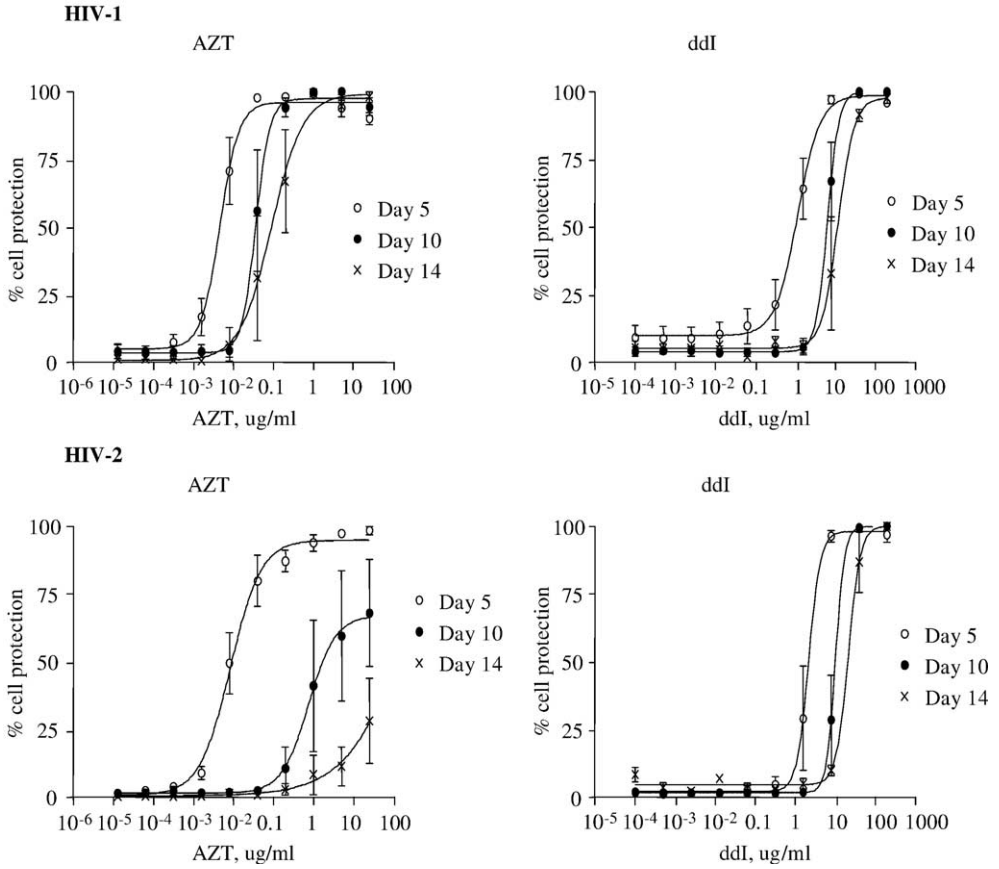


Fig. 5. Inhibition curves for AZT and ddI seen in HIV-1 and HIV-2. Curves were obtained by measuring the percentage of cell protection at increasing AZT concentrations after 5 days of virus replication (standard MT-4/MTT assay) or after 10 and 14 days of virus replication (extended MT-4/MTT assay). Data reflect the mean values observed in 4 HIV-1 (HXB2, $HXB2_{S215}$, CC/H9, and IIIB/H9) and 4 HIV-2 (CDC77618, CBL20/H9, GB122HU, and ROD) isolates.

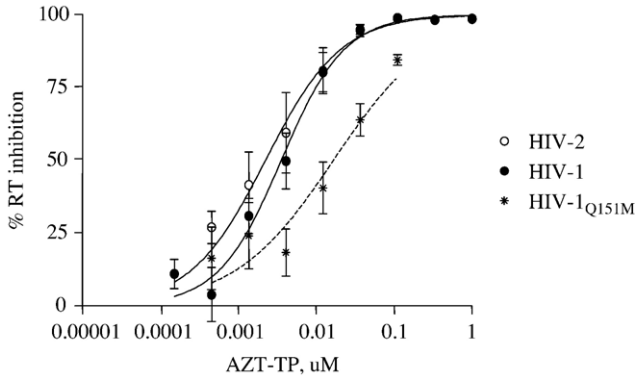


Fig. 6. Enzymatic susceptibility for AZT-TP of HIV-1 and HIV-2. Data show the mean RT inhibition values observed in three HIV-2 isolates (CDC310319, CBL-20/H9, and ROD) and three HIV-1 isolates (HXB2, MN/H9, and LAI) tested in duplicate in two separate experiments. The susceptibility for AZT-TP of a control AZT-resistant HIV-1 mutant carrying the Q151M mutation (L2S.4) is also shown.

for AZT-TP between HIV-1 and HIV-2 RT by using an enzymatic RT assay. An AZT-resistant HIV-1 mutant carrying Q151M (LS2.4) was also evaluated as a control for efficient discrimination (García-Lerma et al., 2000). Fig. 6 compares the susceptibility for AZT-TP seen in three HIV-2 and three HIV-1 isolates. As expected, the control HIV-$1_{Q151M}$ mutant displayed a reduced susceptibility to AZT-TP (4.7-fold) in this assay compared to WT HIV-1. However, the mean $IC_{50}$ for AZT-TP seen in HIV-2 (0.0023 μM) was similar to the mean $IC_{50}$ value seen in WT HIV-1 (0.0036 μM), indicating that HIV-1 and HIV-2 have similar abilities to discriminate against AZT-TP.

*Comparison of baseline RT sequences among HIV-2 and HIV-1*

We next compared the RT sequences (amino acids 37–224) of all the baseline HIV-2 viruses with those of HIV-1. The overall amino acid sequence homology between HIV-1 and HIV-2 RT was 64% (range = 63–65%). Fig. 7 shows that none of the HIV-2 viruses had primary mutations associated with phenotypic resistance to AZT in HIV-1 such as K70R, S215Y/F, or Q151M. However, all the HIV-2 isolates had amino acid changes at positions associated with NRTI resistance in HIV-1 such as codons 69 (T69N), 75 (V75I), 118 (V118I), 210 (L210N), 215 (T215S), and 219 (K219E). Fig. 7 also shows that the 64K and 104K mutations that were observed in HIV-2 isolates GB122HU and CDC77618 during passages with AZT were present at baseline in 3 of the 5 HIV-2 isolates, indicating that 64K and 104K represent natural polymorphisms.

## Discussion

This study represents the first comprehensive analysis of the antiviral activity of AZT on HIV-2. We evaluated the activity of AZT in both primary isolates and laboratory-adapted strains by measuring growth kinetics in the presence of AZT and by assessing the evolution of resistance in selection experiments with AZT. We found that all 5 HIV-2 viruses tested were able to replicate efficiently with a concentration of AZT that was about 3000-fold higher than the $EC_{50}$ value for HIV-1. We also

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                                          69   75                                    118
                  37         47         57         67         77         87         97         107        117        127
HIV-1HXB2         ICTEMEKEGK ISKIGPENPY NTPVFAIKKK DSTKWRKLVD FRELNKRTQD FWEVQLGIPH PAGLKKKKSV TVLDVGDAYF SVPLDEDFRK YTAFTIPSIN
HIV-1HXB2-T215S   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
HIV-1B/H9         .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
HIV-1CC/H9        .......... .......... .......... .......... .......... .......... .......... .......... .....Q.... ..........
HIV-1LAI(BRU)     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
HIV-2CDC77618     ..GK.....Q LEEAP.T... ...T...R.. .KN...M.I. ......V... .T.I...... ....A...RI .......... .I..H....Q .....L..V.
HIV-2CBL-20/H9    ..EK.....Q LEEAP.T... ...T...... .KN...M.I. .....RV... .T.I...... ....A...RI .......... .I..H....Q .....L..V.
HIV-2GB122HU      ..EK.....Q LEEAP.T..F ...T...R.. .KN...M.I. ......V... .T.I...... ....A..RRI .......... .I..H....Q .....L..V.
HIV-2ROD          ..EK.....Q LEEAP.T... ...T...... .KN...M.I. ......V... .T.I...... ....A..RRI .......... .I..H....P .....L..V.
HIV-2CDC310319    ..EK.....Q LEEAP.T... .S.T.....R .KN...M.I. ......V..E .T........ ....AS..RI .......... .I...P...P .....L..V.

                                                                                                  210  215  219
                  137        147        157        167        177        187        197        207        217    224
HIV-1HXB2         NETPGIRYQY NVLPQGWKGS PAIFQSSMTK ILEPFKKQNP DIVIYQYMDD LYVGSDLEIG QHRTKIEELR QHLLRWGLTT PDKKHQKE
HIV-1HXB2-T215S   .......... .......... .......... .......... .......... .......... .......... ........S. ........
HIV-1B/H9         .......... .......... .......... .....R.... .......... .......... .......... .......... ........
HIV-1CC/H9        .......... .......... .......... .....R.... .......... .......... .......... .......F.. ........
HIV-1LAI(BRU)     .......... .......... .......... .....R.... .......... .......... .......... .......F.. ........
HIV-2CDC77618     .AE..K..I. K......... .....YT.RQ V....R.A.. .VILI..... ILIA..RTGL E.DKVVLQ.K EL.NGL.FS. ..E.F..D
HIV-2CBL-20/H9    .AE..K..I. K......... .....YT.RQ V....R.A.. .VILI..... ILIA..RTGL E.DKVVLQ.K EL.NGL.FS. ..E.F..D
HIV-2GB122HU      .AE..K..I. K......... .....YT.RH .....R.A.Q .VI.I..... ILIA..RTDL E.DRVVLQ.K EL.NGL.FS. ..E.F..D
HIV-2ROD          .AE..K..I. K......... .....HT.RQ V....R.A.K .VI.I..... ILIA..RTDL E.DRVVLQ.K EL.NGL.FS. ..E.F..D
HIV-2CDC310319    .AE..K..L. K......... .....Y..G. V.D..R.A.S .VT.I..... IL.A..RSDL E.DKVVSQ.K EL.NNM.FS. .EE.F..D
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Fig. 7. Comparison of RT sequences between baseline HIV-1 and HIV-2 isolates. The amino acids that are associated with AZT resistance in HIV-1 and that were seen in WT HIV-2 isolates are shown in bold.

found absence of mutations in HIV-2 RT during sequential passages with AZT, indicating little or no selective pressure by AZT on these viruses. While some of the isolates showed mixtures at positions 64, 104, 298, 387, and 466, these mutations were frequently seen at baseline in other HIV-2 isolates. However, the impact of these polymorphic residues on the observed AZT resistance of HIV-2 is not known. Taken together, these findings are consistent with a limited antiviral activity of AZT on HIV-2. The opposite was seen with HIV-1, and AZT-resistant HIV-1 mutants were easily selected under the same in vitro conditions and showed patterns of mutations that were consistent with those previously seen (Gao et al., 1992; Larder et al., 1991). Interestingly, despite the need of only 1-nt change to evolve from the WT S215 to 215Y/F, none of the HIV-2 isolates acquired 215Y/F after 10 passages with AZT, while an HIV-1 mutant having S215 acquired 215Y after only 3 passages with AZT. The lack of selection of S215Y/F in HIV-2 strongly supports a low selective advantage of 215Y/F with AZT and is consistent with the low frequency of S215Y/F seen in treated patients (Adje-Toure et al., 2003; Brandin et al., 2003; Descamps et al., 2004; Rodes et al., 2000; van der Ende et al., 2000, 2003).

The selection of K65R and M184I in HIV-2 during passages with AZT and ddI was suggestive of an antiviral activity of ddI on HIV-2. The patterns of mutations seen in vitro were in agreement with clinical data showing K65R and M184I in some HIV-2-infected patients who fail treatment with ddI (Adje-Toure et al., 2003; Brandin et al., 2003; Descamps et al., 2004; van der Ende et al., 2000). The efficient antiviral activity of ddI on HIV-2 was further confirmed by our phenotypic analysis that showed that HIV-2 and HIV-1 were equally susceptible to ddI.

Consistent with previous studies, we found that testing for drug susceptibility by the MT4/MTT assay under conditions used for HIV-1 showed that HIV-2 and HIV-1 were equally sensitive to AZT (Cox et al., 1994; Larder et al., 1990; Witvrouw et al., 2004). However, our demonstration that the conditions of the phenotypic assay are critical to assess the susceptibility of HIV-2 for AZT is significant. We show that high levels of AZT resistance in HIV-2 were only detected when the phenotypic assay was extended from day 5 to days 10–14. We demonstrate that such a dependency on assay conditions was limited to AZT and HIV-2, and that both HIV-1 and HIV-2 had similar $EC_{50}$ values for ddI in the two assay formats. The differences in AZT susceptibility between HIV-1 and HIV-2 by the standard and the modified MT4/MTT assay were not due to an insufficient HIV-2 replication in the absence of drug, since both HIV-1 and HIV-2 showed maximal cytotoxicity at day 5. However, at high AZT concentrations, HIV-1 replication was inhibited while HIV-2 was able to replicate efficiently. The finding that the peak of HIV-2 replication at high AZT concentrations was only seen after 7–14 days of culture is in agreement with the need to extend the MT4/MTT assay to detect AZT resistance. The factors underlying such a delay in replication are not known but may be related to the ability of the HIV-2 isolates to replicate in MT4 cells, or to different susceptibilities of HIV-1 and HIV-2 to changes in dTTP/AZT-TP ratios that might arise during culture of MT4 cells. Additional studies are needed to evaluate these and other possibilities. Our findings emphasize the importance of testing for drug susceptibility by the modified assay for other drugs, particularly for d4T, and highlight the need of studies of selection of resistant mutants to confirm the activity of anti-HIV-1 drugs on HIV-2.

While our in vitro data on the reduced susceptibility of HIV-2 to AZT suggest little drug pressure by AZT in vivo, HIV-2 mutants carrying Q151M and less frequently K70R/S, S215T/Y/F, or E219D have been noted in a few patients treated with AZT or AZT and 3TC (Adje-Toure et al., 2003; Brandin et al., 2003; Descamps et al., 2004; Rodes et al., 2000; van der Ende et al., 2000, 2003). The significance of these mutations and their relationship to AZT susceptibility is not known. Since the natural range of AZT susceptibility among diverse HIV-2 isolates has not been defined, it is possible that some of these mutations may be necessary to enhance AZT resistance in viruses that are naturally less resistant to AZT. Such a possibility is consistent with in vitro findings with NNRTIs where evolution of HIV-2 towards higher levels of NNRTI resistance has been observed despite the inherent baseline resistance of HIV-2 to these drugs (Witvrouw et al., 1999). It is also plausible that some of these mutations may be important for restoring lost AZT resistance. This might be the case, for instance, in HIV-2 mutants containing M184V and Q151M that have been observed in patients treated with AZT and 3TC (Adje-Toure et al., 2003; Brandin et al., 2003; Descamps et al., 2004; Rodes et al., 2000; van der Ende et al., 2000, 2003). In a similar fashion to HIV-1 (Petrella and Wainberg, 2002), the M184V mutation might sensitize HIV-2 to AZT and thus requires the virus to select additional mutations to restore its resistance to AZT. Experimental evidence to support these possibilities will be needed.

Our comparative analysis of AZT susceptibility among HIV-1 and HIV-2 was done in MT-4 cells because of the demonstrated utility of this cell line in evaluating viral replication and drug susceptibility (Pauwels et al., 1988; Vandamme et al., 1999). The direct comparison of several HIV-1 and HIV-2 isolates was essential for demonstrating the phenotypic differences between the two viruses. However, since cell types can influence viral replication and drug susceptibility testing, we expanded our analysis to PBMCs and another established T-cell line. We show further evidence of HIV-2 resistance to AZT by demonstrating that HIV-2 can also replicate efficiently in PBMCs and $CEM_{174}$ cells at high AZT concentrations. Interestingly, replication of HIV-2 in PBMCs was more efficient than our control AZT-resistant HIV-1 mutants carrying K70R, D67N/K70R, or T215Y, suggesting that the levels of AZT resistance in HIV-2 are higher than those seen in these HIV-1 mutants.

Resistance to AZT in HIV-1 is known to occur through two distinct biochemical mechanisms (Scott, 2001). The first mechanism is frequently seen in mutants carrying K70R or T215Y and is explained by an increased excision activity of incorporated AZT by these mutants. A second mechanism is associated with mutations such as Q151M and involves an increased ability of the enzyme to discriminate against AZT-TP (Scott, 2001). Our biochemical data on WT HIV-1 and HIV-2 RT show that both enzymes have similar abilities to incorporate AZT, suggesting that the observed HIV-2 resistance to AZT is not mediated by an increased discrimination against this drug. Therefore, additional biochemical studies that evaluate the excision activity of HIV-2 RT are necessary to determine if this mechanism is involved in the AZT resistance of HIV-2.

The basis of the observed resistance to AZT in HIV-2 is not known. However, our sequence comparison between WT HIV-1 and HIV-2 RT showed important differences among these two enzymes. The finding of T69N, V75I, V118I, and K219E in WT HIV-2 RT was of particular interest since these mutations are all associated with AZT resistance in HIV-1. For instance, T69N confers about 7-fold reduction in AZT susceptibility in HIV-1, and V118I, K219Q, and V75I enhance the levels of AZT resistance of HIV-1 mutants carrying T215Y/F or Q151M (D'Aquila et al., 2003; Winters and Merigan, 2001). However, verifying the possible role of these or other changes in the observed HIV-2 resistance to AZT will require drug susceptibility analysis of site-specific mutants.

In summary, our results indicate that the activity of AZT on HIV-2 is lower than previously thought. The poor antiviral activity of AZT and the fact that currently approved NNRTIs are not effective against HIV-2 emphasize the need for novel antiretroviral drugs specific for HIV-2.

## Materials and methods

### *Viruses and drugs*

The following viruses were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-$2_{CDC310319}$ and HIV-$2_{CDC77618}$ from Dr. Stefan Wiktor and Dr. Mark Rayfield (Owen et al., 1998), HIV-$2_{CBL\text{-}20/H9}$ from Dr. Robin Weiss (Schulz et al., 1990), HIV-$1_{CC/H9}$, HIV-$1_{IIIB/H9}$, and HIV-$1_{MN/H9}$ from Dr. Robert Gallo (Gallo et al., 1984, 1986; Popovic et al., 1984a, 1984b; Shaw et al., 1984), and HIV-$1_{LAI}$ from Dr. Jean-Marie Bechet and Dr. Luc Montaigner (Barre-Sinoussi et al., 1983; Wain-Hobson et al., 1991). HIV-2 isolate GB122HU was kindly provided by Dr. Ron A. Otten, and HIV-2 strain ROD was kindly provided by Dr. Danuta Pieniazek. HIV-2 isolates CDC77618, CDC310319, and GB122HU are primary isolates from AIDS patients and have been propagated solely in primary human PBMCs. HIV-2 CBL-20/H9 and ROD, and HIV-1 CC/H9, IIIB/H9, and LAI/PBMC, are laboratory-adapted strains. HIV-2 CDC77618, ROD, and GB122HU are group A isolates, and HIV-2 CDC310319 is a group B isolate (Barre-Sinoussi et al., 1983; Clavel et al., 1986; Gallo et al., 1986; Owen et al., 1998, 1999; Popovic et al., 1984a, 1984b; Schulz et al., 1990; Wain-Hobson et al., 1991). HIV-1 HXB2, $HXB2_{S215}$, and $L2S.4_{Q151M}$ are recombinant viruses generated by co-transfection of MT-4 cells with an RT-deleted HXB2-based proviral plasmid and cloned RT sequences from HXB2 or isolate L2S as previously described (García-Lerma et al., 2000; Hertogs et al., 1998; Vandamme et al., 1999). The S215 mutation of $HXB2_{S215}$ was introduced by site-directed mutagenesis (García-Lerma et al., 2001a). All virus stocks were aliquoted and maintained at −70 °C. AZT and ddI were obtained through the NIH AIDS Research and Reference Reagent Program. AZT-triphosphate (AZT-TP) was obtained from Moravek Biochemicals (Brea, CA).

### *Analysis of replication capacity in the presence of AZT*

Replication kinetics of HIV-1 and HIV-2 with AZT were measured in MT-4, $CEM_{174}$, and human peripheral blood mononuclear cells (PBMCs). MT-4 and $CEM_{174}$ cells were grown and maintained in RPMI 1640 medium complemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin/streptomycin, and 0.1% sodium bicarbonate. PBMCs were obtained by leukapheresis from an HIV-1-negative blood donor and were cultured in the same media containing 10% interleukin-2 (Roche Diagnostics GmbH, Germany) (Vandamme et al., 1999). Three days prior to the experiment, PBMCs were enriched for $CD4^+$ T cells by removing $CD8^+$ T cells with anti-CD8-conjugated magnetic beads (Dynal, Oslo, Norway) and were activated in complete media containing 0.5 μg/ml of phytohemagglutinin-P (PHA, Difco, Detroit, MI) as described (Cummins et al., 2003).

Replication kinetics in MT-4 and $CEM_{174}$ cells were measured as previously described (García-Lerma et al., 2001a). Briefly, a 500-μl aliquot of HIV-1 or HIV-2 containing 12.5 ng of p24 or p27 antigen was used to infect $1.5 \times 10^6$ MT-4 or $CEM_{174}$ cells. After incubation for 2 h at 37 °C, cells were washed with PBS and resuspended in 10 ml of complete medium containing AZT. Supernatants from each culture were collected at different days. Levels of HIV-1 p24 or HIV-2 p27 antigen were quantified in cell-free supernatants by using the Coulter HIV-1 p24 or SIV p27 antigen assays, respectively, and were used to monitor replication kinetics. The SIV p27 antigen assay provides a sensitive method that can be used to monitor kinetics of HIV-2 replication (Larmoire et al., 1993; Otten et al., 1999). The lower limit of detection of the HIV-1 p24 and the SIV p27 antigen assays were 0.0078 ng/ml and 0.05 ng/ml, respectively. The concentrations of AZT used in experiments done in MT-4 cells were 12.3 μg/ml for cultures done with HIV-2 and 1.6 μg/ml for cultures done with HIV-1. The

concentration of AZT used in experiments done in $CEM_{174}$ cells was 2 μg/ml for both HIV-2 and HIV-1.

A similar approach was used to evaluate the kinetics of virus replication with AZT in PBMCs. Briefly, $1.5 \times 10^6$ activated PBMCs were exposed to each virus stock (12.5 ng of p24 or p27 antigen) for 2 h at 37 °C. After washing with PBS, cells were resuspended at $2 \times 10^6$ cells/ml in complete media containing AZT (2 μg/ml). 200-μl cultures were done in quadruplicate using 96-well plates (Costar), and supernatants (100 μl) were collected at different days followed by the addition of complete media with AZT. At day 7 of the experiment, $2 \times 10^5$ PHA-stimulated PBMCs were added to each culture and cultures were maintained for an additional 7 days.

*In vitro selection of drug resistance*

Selection of drug resistance was done by sequential passages of the isolates in the presence of increasing concentrations of AZT or AZT and ddI as described previously (García-Lerma et al., 2001a). Briefly, a 500-μl aliquot of HIV-1 or HIV-2 containing 25 ng/ml of p24 or p27 antigen, respectively, was added to $1.5 \times 10^6$ MT-4 cells and cultured in the presence of AZT (0.024 μg/ml) or AZT and ddI (0.003 μg/ml and 2 μg/ml, respectively). Cultures were then incubated at 37 °C and media containing AZT or AZT and ddI were changed every 3–4 days as required. Virus production was monitored by microscopic assessment of syncytium formation. Once virus production was evident through all the culture, 500 μl of clarified supernatant was added to $1.5 \times 10^6$ fresh cells and cultured with the same or a higher concentration of drug/s. The range of AZT and ddI concentrations used in the selection experiments is detailed in Tables 1 and 2. Genotypic changes in the RT were monitored at selected passages by sequence analysis as described below.

*Analysis of the susceptibility to AZT and ddI in HIV-1 and HIV-2*

The susceptibility of HIV-1 and HIV-2 to AZT and ddI was measured using the MT-4/MTT assay as previously described (Pauwels et al., 1988; Vandamme et al., 1999). Briefly, MT-4 cells ($3 \times 10^4$) were exposed to 150 $CCID_{50}$ of each virus in triplicate both in the absence and in the presence of serial dilutions of AZT or ddI. Control uninfected MT-4 cell cultures were also included and were used to correct for drug toxicity. In the standard format of the assay, the concentration of drug required to inhibit 50% or 90% virus-induced cell killing (50% or 90% effective concentration; $EC_{50}$ and $EC_{90}$, respectively) was calculated after 5 days in culture by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase of metabolically active cells (Pauwels et al., 1988; Vandamme et al., 1999).

$EC_{50}$ and $EC_{90}$ values were also determined using an extended MT4/MTT assay format that allows for additional rounds of viral replication in the presence of drug. In this format, a 40-μl aliquot of each culture was taken at day 5 and transferred to a new 96-well plate containing complete media with or without drug. $EC_{50}$ and $EC_{90}$ values were then re-calculated after 5 additional days of culture (day 10). The same procedure was done at day 10 and $EC_{50}$ and $EC_{90}$ values were re-calculated at day 14. During the 14 days of culture, the range of AZT or ddI concentrations remained constant. Changes in $EC_{50}$ values >3-fold are considered significant (cut-off point) (Witvrouw et al., 2004).

*Enzymatic susceptibility of HIV-2 RT to AZT-TP*

Susceptibility to AZT-TP of virion-associated RTs was measured using a non-radioactive microtiter plate-based RT assay (Roche Diagnostics GmbH, Germany) as described previously (García-Lerma et al., 2003). Briefly, viruses normalized by their levels of RT activity were exposed for 30 min to serial 3-fold dilutions of AZT-TP prepared in lysis buffer. RT reaction was done for 2 h at 37 °C in an RT buffer containing poly(A) × $oligo(dT)_{15}$ template/primer, digoxigenin-dUTP, biotin-dUTP, and dTTP. Levels of RT activity were quantified by using an ELISA-based chemiluminescent assay according to the manufacturer's instructions. Percentage of RT inhibition was determined by dividing the signal obtained in reactions done with AZT-TP with that seen in reactions done in the absence of drug; $IC_{50}$ values were calculated as previously described (García-Lerma et al., 2003).

*RT-PCR and sequence analysis*

RT sequences of HIV-1 and HIV-2 were obtained from amplified RT-nested PCR products using an ABI377 automated sequencer and were analyzed using the Vector NTI program (Version 7, 2001). Briefly, RNA was extracted using the QIAmp Viral RNA kit (Qiagen). For HIV-1, the RT reaction was done for 1 h at 42 °C using primer RT2 as described previously (García-Lerma et al., 2000). After a first round of PCR amplification using primers RT1 and RT2, 4 μl was subjected to a second round of amplification using primers A35 and NE1. Primers AV36, AV44, A35, and NE(1)35 were used for sequence analysis (amino acids 32–224) (Schmit et al., 1996; Vandamme et al., 1999). For HIV-2, the RT reaction was done for 1 h at 42 °C using primer RT2-2 (5′-GAA GTC CCA GTC TGG GAT CCA TGT CAC TTG CCA-3′). After a first round of PCR amplification using primers RT2-2 and PR1 (5′-GGG AAA GAA GCC CCG CAA CTT C-3′), 4 μl was subjected to a second round of amplification using primers RT7 (5′-GGG CCA AAG A(C)TG(C) AGA CAA TGG CC-3′) and RT8 (5′-TTG GCC AC(G)A GTT CAT AA(G)C CCA TCC-3′). Primers RT7, RT8, RT9 (5′-CAG TAA ACA ATG CT(A)G AAC CAG GAA-3′), and RT10 (5′-G T(G)GA TCC CTT CCA

TCC C(T)TG TGG-3′) were used for sequence analysis of HIV-2 RT (amino acids 32–224).

Amino acids 225–550 of HIV-2 RT were also sequenced in baseline isolates and in isolates collected at selected passages with AZT. Briefly, RNA was extracted using the QIAmp Viral RNA kit and the RT reaction was done for 1 h at 42 °C using primer IN R1 (5′-TGG GCA CAK GTG TTT ACT ATC TG-3′). After a first round of PCR amplification using primers INR1 and RT11 (5′-ATC YTA ATR GCT AGT GAC AGG A-3′), 5 μl was subjected to a second round of amplification using primers RT12 (5′-GAG AAG TTC CAA AAR GAC CTC C-3′) and RT13 (5′-TGT CTR ATG CCY TGA CTT ACT A-3′). Primers RT12, RT13, RT14 (5′-AAG TCC CAW TCT GGG ATC CAT G-3′), and RT15 (5′-ACC TGG GAR CAG TGG TGG GAT A-3′) were used for sequence analysis. Nucleotide RT sequences (amino acids 32–550) of baseline HIV-2 isolates CDC77618, CDC310319, GB122HU, ROD, and CBL20/H9 and of isolates collected at passage 10 with AZT have been deposited in the GenBank database (accession numbers AY965902–AY965911).

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