In vitro analysis of human immunodeficiency virus type 1 resistance to nevirapine and fitness determination of resistant variants

Maria Dolores Iglesias-Ussel, Concepción Casado, Eloísa Yuste, Isabel Olivares and Cecilio López-Galín dez

1 Servicio de Virología Molecular, Centro Nacional de Biología Fundamental, Instituto de Salud Carlos III, Carretera Majadahonda-Pozuelo Km. 2, Majadahonda, 28220 Madrid, Spain
2 Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

Nevirapine-resistant variants were generated by serial passages in MT-2 cells in the presence of increasing drug concentrations. In passage 5, mutations V106A, Y181C and G190A were detected in the global population, associated with a 100-fold susceptibility decrease. Sequence analysis of biological clones obtained from passage 5 and subsequent passages showed that single mutants, detected in first passages, were progressively replaced in passage 15 by double mutants, correlating with a 500-fold increase in phenotypic resistance. Fitness determination of single mutants confirmed that, in the presence of nevirapine, every variant was more fit than wild-type with a fitness order Y181C > V106A > G190A > wild-type. Unexpectedly, in the absence of the drug, the Y181C resistant mutant was more fit than wild-type, with a fitness gradient Y181C > wild-type > G106A > V190A. Using a molecular clone in which the Y181C mutation was introduced by in vitro mutagenesis, the greater fitness of the Y181C mutant was confirmed in new competition cultures. These data exemplify the role of resistance mutations on virus phenotype but also on virus evolution leading, occasionally, to resistant variants fitter than the wild-type in the absence of the drug.

Introduction

Human immunodeficiency virus type 1 (HIV-1) and all RNA viruses have adopted as an evolutionary strategy an error-prone mechanism of replication. This results in the accumulation of mutations in every virus replication. In general, the occurrence of mutations is a random process and consequently most of mutations are deleterious. In RNA viruses the accumulation of deleterious mutations during replication is responsible for the Muller ratchet effect (Muller, 1964), which has been described for many RNA viruses (Chao, 1990; Escarmís et al., 1996), including HIV-1 (Yuste et al., 1999). However, some mutations that appear during the continuous replication of RNA viruses could offer some adaptive advantage, like natural antiviral-resistance mutations (Nájera et al., 1994, 1995).

Potent antiviral treatment has brought about a breakthrough in the AIDS epidemic in developed countries. The principal target for anti-HIV therapy has been reverse transcriptase (RT). HIV-1 RT inhibitors can be divided into two groups (De Clercq, 1992): nucleoside analogues (Fischl et al., 1987; Mitsuya et al., 1985) and non-nucleoside RT inhibitors (NNRTI), like nevirapine (NVP). As opposed to what occurs with nucleoside analogues (Larder et al., 1989, 1991a), viruses resistant to NNRTI arise quickly in vivo (Richman et al., 1994) and in vitro (Balzarini et al., 1993; Larder et al., 1987; Nunberg et al., 1991). The loss of antiviral activity is associated with the acquisition of several mutations around the hydrophobic catalytic pocket in the p66 subunit of the RT, mainly at amino acid positions 103 (K → N), 106 (V → A), 181 (Y → C/I), 188 (Y → C) and 190 (G → A) (Kohlstaedt et al., 1992; Korber et al., 1998; Smerdon et al., 1994). Some of these alterations have an effect not only on the resistant phenotype but also on enzymatic properties (Tantillo et al., 1994).

One of the consequences of the error-prone replication of RNA viruses is the existence in any HIV-1 virus population of a swarm of related genomes (Meyerhans et al., 1989; Sabino et al., 1994; Wain-Hobson, 1993), termed quasispecies, prepared for the rapid dominance of different variants in response to
different environments. These variants display different phenotypic and genotypic characteristics and they compete for the prevalence of the most fit variant (designated wild-type virus) in the virus population by Darwinian selection. The parameter that better measures the dominance of one virus is virus fitness and, in certain conditions, is related to the presence of a mutant in a virus population (Coffin, 1995). Recently there have been interesting reports implicating virus fitness of the different HIV-1 antiviral-resistant variants in vitro with its dominance in virus population within patients (de Ronde et al., 2001; Goudsmit et al., 1996, 1997; Harrigan et al., 1996).

We report the genotypic and phenotypic characterization of HIV-1 NVP-resistant variants selected by in vitro passage of a clonal wild-type virus to increasing amounts of drug. The evolution of the distinct single and double mutants present in the virus population was examined during the passages. To further study the role that each single resistance mutation plays in antiviral resistance, we carried out a fitness study of single resistant variants, in both the presence and absence of the antiviral.

Methods

**Virus and cells.** Virus used in the generation of NVP-resistant variants (V61) was obtained from the infectious molecular clone 89E601 (Oliffares et al., 1997, 1998), derived from an HIV-1 Spanish isolate (Sánchez-Palomino et al., 1993), by electroporation of the plasmid in Cos-1 cells and coculture with MT-4 cells. The RT sequence and the susceptibility to NVP of this virus were determined and corresponded to a drug-sensitive isolate (Oliffares et al., 1998).

An MT-2 cell line (Miyoshi et al., 1981) was used for the in vitro selection of HIV-1 NVP-resistant variants, for the evaluation of the replicative capacity of the selected virus, for susceptibility testing and for the competition assays. An MT-4 cell line (Miyoshi et al., 1981) was used in a plaque assay to obtain biological clones. Both cell lines are maintained in RPMI 1640 (Bio-Whittaker) supplemented with 10% foetal bovine serum (Gibco) plus 1% antibiotics (Bio-Whittaker). HT4-6C, HeLa cells expressing the human CD4 receptor (Chesebro & Wehrly, 1988), were used to determine drug susceptibility. This cell line is maintained in Dulbecco’s modified Eagle’s medium (Gibco) containing 10% foetal bovine serum plus antibiotics (DME-10 or growth medium).

**Test compound.** NVP (11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido[3,2-b:2,3’-e][1,4]diazepin-6-one) stock solution was stored at −20 °C until use.

**In vitro selection of HIV-1 NVP-resistant variants.** V61 was passaged in 5 × 10^6 MT-2 cells in the presence of increasing concentrations of drug (Table 1) at an m.o.i of 0.01, following previous protocols (Gao et al., 1992, 1994; Larder et al., 1991a, 1993). In order to obtain adequate intracellular levels of drug, cells are pre-incubated for 4 h. New medium containing the appropriate drug concentrations was added every 2 days to cultures. Passages were performed with the supernatant of the preceding passage, taking into account that virus titres do not change significantly during passages (Sánchez-Palomino et al., 1993).

**Biological characterization of the resistant variants.** Virus titre assays were performed in MT-2 cells and titres, expressed as 50% tissue culture infective dose (TCID₅₀), were calculated manually by the Spearman-Karber method (Mascola, 1999). Susceptibility to NVP, expressed as 50% inhibitory concentration (IC₅₀), was determined by Hela CD4 plaque assay, or by the MTS method. Quantitative plaque reduction assay in Hela CD4 (HT4-6C) cells was carried out as described (Larder et al., 1989, 1990). Briefly, 4 × 10⁶ cells were seeded in 24-well tissue culture plates (Falcon) and inoculated with 100–200 p.f.u. of virus in 0.2 ml DMEM-5 and adsorbed for 1 h at 37 °C. Then five different 1:10 dilutions of NVP were added to duplicate wells in 0.8 ml growth medium. Four days later monolayers were fixed with 100% methanol and stained with 0.25% crystal violet to visualize plaques. The colorimetric method is based on the reduction of tetrazolium to formazan (Promega) in viable cells. The assay was carried out in 1 × 10⁶ MT-2 cells with 100 TCID₅₀ per well and 5–6 different concentrations of antiviral and performed in triplicate. Four days later, 20 µl of the MTS reagent was added, incubated for 4 h at 37 °C and read at 490 nm. In both cases, the percentage of reduction of the effect was calculated by comparison with a drug-free control. The dose–response curves are obtained when these percentages are plotted against the logarithm of drug concentrations. The IC₅₀ was determined by linear regression using the median effect equation (Chou & Talalay, 1984), with the help of GraphPad Prism software (2.01 version, 1996). Resistance is defined as an IC₅₀ greater than 100-fold of the initial value (López-Galindo & Guerra Romero, 1997).

**Biological cloning.** Biological clones were obtained using an agarose MT-4 plaque assay as previously described (Harada et al., 1987; Sánchez-Palomino et al., 1993). Well isolated plaques were picked at random 7 to 10 days post-infection and resuspended in 300 µl of culture medium (Yuste et al., 1999). Virus stocks from NVP-resistant clones were obtained by infecting 5 × 10⁶ MT-2 cells in the presence (for resistant clones) or absence (for wild-type virus) of low concentrations of drug (0.25 mM).

**Genetic analysis of resistant variants.** Variants obtained by in vitro selection were analysed from HIV-1 proviral DNA of infected cells. Total cellular DNA from infected cells was extracted by phenol–chloroform followed by ethanol precipitation (Perucho et al., 1987) for large amounts of cells, or by the Instagene purification matrix (Biorad) for smaller numbers of cells. A 1951 bp fragment including the complete polymerase domain of the RT in the pol gene was amplified by PCR as described previously (Nájera et al., 1994, 1995) using Taq polymerase (Perkin Elmer). Primers 54RU (5’ ATGGTGCCAGGAGATGGAAACCA 3’, positions 1719–1742), where the number corresponds to the position in the BH10 genome (GenBank accession no. M15654), and 53RD (5’ GGCAGATTCTAATGCTGTTCCAC 3’, positions 3642–3669) were used. DNA from biological clones was amplified by using a nested PCR, performed with RT oligonucleotides 5RU and 20RD (Nájera et al., 1994, 1995). PCR products were purified with the High Pure PCR product purification kit (Promega) and sequenced with the fmo1 DNA cycle sequencing system (Promega), with primers 14RD (Nájera et al., 1994, 1995) or 15R (5’ GATATATGAAATCAATGCTGTTCCAC 3’, positions 2333–2358) according to the manufacturer’s instructions.

**Generation of mutant virus Y181C.** In order to obtain the mutant Y181C virus, in vitro mutagenesis was performed in the infectious molecular clone 89E601 using the Quikchange site-directed mutagenesis kit (Stratagene). Mutagenesis was carried out in the pT1FA subclone (Oliffares et al., 1997) with primers 283RU (5’ CCAGACA-TAGTTATCTGTCAATACATGGACG 3’, positions 2433–2463) and 283RD (complementary to above). The mutant Y181C virus was obtained by electroporation of the mutated plasmid in COS-1 cells as previously reported (Oliffares et al., 1997) and grown in MT-4 cells to obtain a stock. Mutation was confirmed by nucleotide sequencing of the
Table 1. Description of the *in vitro* selection procedure and drug concentrations used to generate resistant viruses and phenotypic characteristics of the variants

<table>
<thead>
<tr>
<th>Passage</th>
<th>NVP conc (µM)</th>
<th>Duration of passage (days)*</th>
<th>+NVP Titre (TCID₅₀)†</th>
<th>IC₅₀ (µM) (fold resistance)‡</th>
<th>IC₁₀ (µM) (fold resistance)§</th>
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<tr>
<td>0</td>
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<td>-</td>
<td>6.4 x 10⁴</td>
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<td>1</td>
<td>0.05</td>
<td>8</td>
<td>9</td>
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<td>4</td>
<td>0.2</td>
<td>9</td>
<td>6</td>
<td>-</td>
<td>0.6 (6)</td>
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<tr>
<td>5</td>
<td>0.4</td>
<td>16</td>
<td>6</td>
<td>1.6 x 10⁵</td>
<td>&gt; 10 (&gt; 100)</td>
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<tr>
<td>6</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>-</td>
<td>9 (90)</td>
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<td>1</td>
<td>7</td>
<td>5</td>
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<td>6</td>
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<td>1.2 x 10⁴</td>
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<td>11</td>
<td>8</td>
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<tr>
<td>15</td>
<td>64</td>
<td>4</td>
<td>1.7 x 10⁴</td>
<td>&gt; 50 (&gt; 500)</td>
<td>&gt; 100 (&gt; 500)</td>
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</table>

* Infections were performed at the same time in the presence or the absence of drug.
† Determined by cytopathic effect in MT-2 cells in two determinations.
‡ Determined by the HeLa CD4⁺ plaque reduction assay.
§ Determined by the MTS colorimetric assay.
– Not determined.

In *in vitro* determination of relative fitness. Fitness determination was performed by growth competition experiments as previously described (Chao, 1990; Escarmis et al., 1996; Holland et al., 1991; Yuste et al., 1999). Cultures between the clone to be tested and the wild-type virus were carried out at an initial proportion of 1:1 for five passages and in three independent experiments. 5 x 10⁴ MT-2 cells were infected at a m.o.i of 0.05, both in the absence and in the presence of 0.25 µM NVP. For the next passage, fresh MT-2 cells were infected with 5 µl supernatant of the preceding passage. RNA was extracted from 20 µl culture supernatant as described (Boom et al., 1990), with a guanidinium isothiocyanate lysis buffer and glass milk, and resuspended in 50 µl TE buffer. After isolation of viral RNA, 10 µl of this RNA preparation was reverse-transcribed and amplified using the Titan one-tube RT–PCR system (Boehringer Mannheim) with primers 226RU (5’ CAGTCCCCCTTAGATAAGAA-TGGAGAAGTACACTGC 3’, positions 2257–2293) and 250RD (5’ CCATTITACAGGATGGAGTCTCCCAACCCATCAAAGG 3’, antisense, complementary to positions 2588–2625) in the first PCR and primers 15’RU and 248RD (5’ GATGGAATCATACATACACCTTACAATTAAGGAATGGAGG 3’, antisense, complementary to positions 2580–2615) in the second PCR. cDNA of a wild-type clone obtained in the second PCR was labelled with [α-³²P]dATP (3000 Ci/mmole) in an asymmetric PCR using primer 15’RU. About 10000 c.p.m. of this radioactive PCR probe was mixed with 50–100 ng of unlabelled second-round PCR product from the competing viruses in annealing buffer (0.1 M NaCl, 10 mM Tris–HCl pH 7.8 and 2 mM EDTA). The DNA mixtures were denatured at 94 °C for 2 min and then quickly cooled. Heteroduplexes were then resolved on a denaturing 15% polyacrylamide–15% urea gel in TBE (88 mM boric acid and 2 mM EDTA) at 15 mA for 18 h. Autoradiograms were obtained by exposing gel on a Fuji 2000 instrument for 2 h. The quantification of the ratio of the wild-type cDNA (homoduplex) to the cDNA of the mutant virus (heteroduplex) was determined by densitometry with the help of the PCBAS program.

Fitness vectors were derived from the ratio of the competing clone to the wild-type virus in each passage in relation to the initial proportion (Rn/Ro), as in previous reports (Chao, 1990; Escarmis et al., 1996; Holland et al., 1991; Yuste et al., 1999). The vector was obtained by linear regression and the slope represents the relative fitness value of the corresponding virus clone. In this procedure the vector of the reference clone is established as zero (relative fitness 1), a negative value represents a virus less fit than the wild-type and positive value a more fit virus.
Results

In vitro generation and genotypic characterization of NVP-resistant variants

In order to study the generation and dynamics of antiviral resistance, a wild-type virus was serially passaged in MT-2 cells in the presence of increasing amounts of an NNRTI. For the in vitro selection, NVP was used because of its high antiviral activity as well as the rapid detection of resistant variants in vitro (Richman et al., 1991) and in vivo (Richman et al., 1994). Antiviral concentrations used in each passage are summarized in Table 1. At passage 5, virus infection was delayed 10 days in relation to the control. To study this delay, the viruses obtained from this and subsequent passages were subjected to genetic characterization in the pol gene. Consensus sequence revealed different mixtures of nucleotide corresponding to wild-type virus and NVP-resistant mutations at codons V106A (GTA → GCA), Y181C (TAT → TGT) and G190A (GGA → GCA) since the fifth passage. Sequence analysis of 10 biological clones from passages 5, 6, 10 and 15 showed six distinct resistant genotypes and the wild-type (Fig. 1a). At passage 5, only two variants were detected, both displaying single mutations: Y181C, in 70% of the clones, and V106A in 30%. Due to the low number of clones analysed G190A was not detected in the quasispecies. The same single mutants with different proportions were rescued in passage 6, but also the G190A variant in 20% of the clones and one double V106A-G190A variant appeared in 10%. In passage 10 the Y181C (60%) and G190A (20 %) mutants were present together with a Y181C-G190A double mutant. It is interesting to note the detection of a wild-type genotype at this passage. Finally, at passage 15, the double mutant Y181C-G190A (70%) and a new one, V106A-Y181C (20%), were the major forms but the single Y181C mutant was still evident (10%). This passage showed a 7 day delay in infection time.

Phenotypic analysis of virus recovered by passage in NVP

Susceptibility to NVP of virus selected was determined using a quantitative plaque reduction assay in HeLa CD4+ cells (Larder et al., 1989, 1990), as described in Methods. As expected from genetic data, the phenotypic analysis of viruses from passages 5, 6 and 10 corresponded to resistant variants (IC50 > 10, 9 and 8 µM, respectively), with a 100-fold decrease in susceptibility. In the last passage the increase of resistance was > 500-fold (with an IC50 50 µM) in HeLa CD4 cells and an IC50 > 100 µM in the MTS assay, coincident with the major presence of double variants (Table 1). Phenotypic analysis of the Y181C virus obtained by in vitro mutagenesis was carried out as indicated in Methods, giving a highly resistant IC50 concentration of 573 µM versus an IC50 concentration of 1.51 µM for the corresponding wild-type virus.

Fitness determination

In order to further characterize the single resistant variants virus fitness was determined. Trying to minimize the biological and virus differences among variants, clones with V106A, Y181C and G190A mutations were derived from the same passage (number 6). Clone 6 displayed the V106A change, clone 12 the Y181C mutation, and clone 13 the G190A transition. A wild-type clone from the control culture of V61 was obtained from the same culture passage and it was used as the reference virus in the competition experiments. Fitness estimation was first evaluated by looking at the dominance of the virus in the competition experiments. This analysis provided a fitness order among the variants, which was the same in the three
\textit{In vitro} fitness of NVP-resistant mutants

**Fig. 2.** Fitness vectors of NVP-resistant clones. Fitness values were determined from five competition passages between wild-type and the resistant clones, as described in Methods. These competitions were repeated three times with similar results but only one experiment is shown. The proportion of every virus was quantified by densitometry of the mutated nucleotide in the sequencing autoradiogram as indicated in Methods. The ratio between the presence of the two viruses in every passage (Rn/Ro) is represented by solid squares and used to build fitness vectors. The linear regression equation as well as goodness of fit (R^2) is shown. Competition experiments were performed in the presence of 0–25 µM NVP (a) or in the absence (b) of the inhibitor.

**Fig. 3.** Fitness analysis of the Y181C mutant. Mutant Y181C virus was obtained by \textit{in vitro} mutagenesis as indicated in Methods. Fitness values were determined from competition cultures between the mutant and the wild-type virus during five passages in the presence (0–25 µM) and in the absence of NVP, as described in Methods. The competitions cultures were performed in triplicate. (a) HTA pattern obtained in the five competition passages using wild-type virus as a probe. Homoduplex band (Ho) represents the proportion of the wild-type virus whereas the proportion of the mutant Y181C is represented by the heteroduplex band (Hx). (b) Fitness vectors in the presence or the absence of the drug. Each point represents an average of the values obtained in the three replicas performed between the proportion of the resistant virus to the wild-type (Rn/Ro) in the passage in relation to the proportion in the initial mixture. The linear regression equation as well as goodness of fit (R^2) and P values are shown.

Independent experiments. For more detailed studies the relative fitness values were established from vector slope, obtained as indicated above, and the result of one of these competition experiments is shown in Fig. 2. Fitness values of all three resistant variants in the presence of drug gave values higher than the wild-type clone and the relative fitness order was: Y181C > V106A > G190A > wild-type. In the absence of the drug, the Y181C mutant displayed a fitness value higher than the wild-type and fitness was Y181C > wild-type > V106A ≧ G190A. Values generated in the three
competition experiments were not identical but the fitness order of the variants was always maintained.

To confirm the fitness value of the Y181C mutant in the absence of the drug, competitions experiments between wild-type and mutant virus were performed with a molecular clone in which Y181C was introduced by in vitro mutagenesis as indicated in Methods. Quantification of the variants was carried out by HTA as described in Methods. The fitness vectors are shown in Fig. 3. As can be seen the fitness of the Y181C mutant virus was greater than the wild-type both in the absence \( R^2 = 0.89, P = 0.0045 \) and in the presence of the drug \( R^2 = 0.99, P = 0.011 \), Fig. 3). These experiments, performed in triplicate, confirmed that the fitness value of the Y181C mutant was higher than the wild-type in the absence of NVP.

**Discussion**

In the present in vitro study designed for the analysis of NVP resistance, a wild-type virus, V61, derived from a molecular infectious clone (Olivares et al., 1997) was used, in order to minimize the possibility of the pre-existence of natural resistance mutations. The first and most prevalent variant detected until late passages was Y181C. It confers a high phenotypic resistance (Nunberg et al., 1991; Richman et al., 1991; Spence et al., 1996), but by culture 15 it was replaced by double variants which included the Y181C alteration. However we observed the emergence of five other mutants: two single, V106A, G190A, and three double, Y181C-G190A, V106A-Y181C and V106A-G190A. The G190A mutation in a wild-type virus and the double V106A-G190A mutant were detected for the first time in vitro. We have observed a delay in the appearance of cytopathic effect in relation to the control at passage 5, coincident with the dominance of single resistant mutants as well as in passage 15 when double mutants prevail in the population.

Phenotypic assays performed in HeLa CD4+ cells and MTS assays showed a 100-fold reduction in NVP susceptibility from passage 5. Mutations detected in RT from this passage have been demonstrated to confer NVP resistance by site-directed mutagenesis (Richman et al., 1991). At passage 15 an increase in IC\(_{50}\) values from 0.1 to above 50 µM in HeLa assay or from 0.2 to above 100 µM using the MTS method (≥ 500 x) (Table 1) was coincident with the major presence of double variants. In general, successive accumulation of mutations caused an increase in phenotypic resistance (Larder et al., 1991b). The appearance of double mutants in our study could be the result of the incorporation of new mutations into single mutants or to recombination between single mutants, as previously described in the development of resistance to RT inhibitors (Gu et al., 1995; Kellam & Larder, 1995; Moutouh et al., 1996). However, we could not discriminate between these two possibilities because of the lack of specific genetic markers. Studies with other antivirals have shown that in vivo and in vitro resistance is a progressive process, associated with the accumulation of different mutations in the RT region of the pol gene (Boucher et al., 1992; Moutouh et al., 1996). These data reflect the existence within patients of multiple competing resistant variants (Richman et al., 1994; Havlir & Richman, 1996), which results in the prevalence of the most fit virus as a function of the resistant phenotype and other virus factors.

Fitness values obtained from NVP-resistant viruses V106A and G190A displayed relative fitness lower than the wild-type in the absence of drug, as anticipated. In contrast, fitness value obtained with the Y181C NVP-resistant variant in the absence of the drug was greater than the wild-type virus. This result was somewhat unexpected and was in contrast with general concepts which relate wild-type virus with the best adapted and most fit variant present in any environment (Eigen & Biebricher, 1988). In the absence of the drug, any resistance mutation is expected to have a negative effect on fitness, the resistance cost (Havlir & Richman, 1996), and resistant mutants replicate more poorly than wild-type (Coffin, 1996b). Why this mutation is not present as the wild-type if it confers a fitness advantage to the virus? This could be related to the need for a threshold level for a variant to prevail in a virus population (de la Torre & Holland, 1990), to clonal interference (Miralles et al., 1999) or to frequency-dependent selection (Ayala, 1971). The fact that the Y181C mutant displays a positive fitness in support of different evidence. The cost of resistance mutations is generally low, as shown by the maintenance of resistant variants without obvious reversion during repeated passages in the absence of drug (Borman et al., 1996; Gao et al., 1992) but also in patients that interrupted NVP treatment, even after 20 months without therapy (Havlir & Richman, 1996). In vitro we detected the appearance of the Y181C mutation in an AZT-resistant virus after 11 passages in the absence of the drug, which became the major form by passage 16 (Najera et al., 1994). Also, the Y181C residue is found naturally in HIV-2 viruses (Shih et al., 1991) and in isolates of subtype O (Descamps et al., 1997; Quiñones-Mateu et al., 1997). Also, it has been estimated that the Y181C resistant variant was present in around 7 to 133 per 10000 copies of HIV RNA in plasma from patients before drug treatment (Havlir et al., 1996). Similarly, there are recent reports on the superior fitness value of the Q151M multiresistant variant in the absence of drugs (Kosalaraksa et al., 1999).

The positive value of fitness of the Y181C virus (Fig. 2) is small but, due to the very high number of replication cycles in HIV-1, this increase in fitness could suffice for the dominance of the variant in virus populations (Coffin, 1996a). Fitness values are always relative to the system in which they are determined, generally in vitro conditions (i.e. T cell lines). These fitness values cannot be directly translated to other systems, particularly to in vivo situations, in which different cell lines and changing environments are operating.

In summary, by in vitro culture of a wild-type virus we have obtained NVP-resistant variants with different combinations of
resistant mutations. Single resistant mutants were obtained at early passages but were replaced by double mutants at passage 15. Regarding virus fitness, we have found that the Y181C mutant is more fit than wild-type virus in the presence of the drug. This result shows that resistance mutations not only give an advantage for the resistant phenotype but also could give a better fitness to the virus with a positive effect on virus evolution.

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