Original article

Competitive capacity of HIV-1 strains carrying M184I or Y181I drug-resistant mutations

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Background Virus with nucleoside reverse transcriptase inhibitors (NRTIs) or nonnucleoside reverse transcriptase inhibitors (NNRTIs) resistant mutations show different evolution tendencies when the anti-viral therapies are interrupted. Understanding the replication fitness of drug-resistant virus is important for the study of the prevalence of drug-resistance. For this purpose, we characterized the replication capacity of HIV-1 virus carrying lamivudine (3TC) or nevirapine (NVP) resistant mutations.

Methods 3TC and NVP resistant variants were induced *in vitro* by selecting wild type virus in the presence of drugs. For the competitive replication assay, drug-resistant variants were cocultured with wild-type virus in the presence or absence of drugs. The ratios of the viral species were determined over time by using a real-time RT-PCR-based assay.

Results 3TC-resistant (M184I mutation) and NVP-resistant (Y181I mutation) virus should be selected *in vitro* in two different ways. The competitive replication assay showed that the ratio of virus carrying a M184I mutation increased from 98.8%, while the wild type virus decreased to 1.2% after 4 passages in the presence of 3TC; the percentage of virus carrying the Y181I mutation increased to 90.5%, while wild type virus decreased to 9.5% in the presence of NVP. In the absence of drugs, the ratio of virus carrying the M184I mutation decreased to 5.3%, while wild type virus increased to 94.7%; the ratio of virus carrying Y181I increased to 75%, while wild type virus decreased to 25% after 4 passages.

Conclusions The NVP-resistant virus is fitter than wild type virus even in the absence of NVP that may be the reason that NNRTIs-resistant virus is spreading quickly.

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Viral replication fitness implicates the replication capacity of virus in a specific environment.[1] It was supposed that strains with the highest replication capacity would become the dominant strains in specific situations.[2] Drug-resistant mutations make virus suitable for viral replication under drug pressure, however, the emergence of drug resistance sometimes reduces the inherent ability of HIV to replicate (replicative capacity). When anti-viral therapy was implemented, drug-resistant strains always replicate better than wild type virus under selective pressure.[3] As a result, drug-resistant virus will replace wild type virus and become dominant. Once anti-viral therapy is interrupted, some wild type viruses *in vivo* can have a higher replication capacity and drug-resistant virus replace the dominant strain.[4]

Change of replication fitness caused by drug-resistant mutations is important for the investigation of HIV-1 pathogenesis. In a previous cohort study of HIV drug-resistance, we found that virus with nucleoside reverse transcriptase inhibitors (NRTIs) or nonnucleoside reverse transcriptase inhibitors (NNRTIs) resistant mutations showed different evolution tendencies when the anti-virus therapies were interrupted. Without drug pressure, virus with NRTIs resistant mutations grew slowly and was quickly replaced by wild type virus; however, virus with NNRTIs resistant mutations could persist *in vivo* for a long time.[5] In order to elucidate the mechanism, we studied the replication fitness of two HIV strains with a typical NRTIs resistant mutation (M184I) and a typical NNRTIs resistant mutation (Y181I) *in vitro*.

METHODS

Materials

The wild type drug-sensitive HIV-1 strain CNHN24 was isolated in 2001 from the blood mononuclear cells (PBMCs) of a 33-year-old female AIDS patient living in Henan Province of China. The full length genome of the virus has already been sequenced and submitted to Genebank with accession number AY180905. The subtype of the virus was B', prevalent in Henan and Heilongjiang provinces in China.[6] MT-4 cells were used for replication of the virus and grown in RPMI1640 medium (Gibco, USA) supplemented with 10 U/ml

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penicillin, 50 μg/ml gentamycin and 10% fetal bovine serum. Heptodin containing 100 mg lamivudine (3TC) per tablet was produced by GlaxoSmithKline Company (England). One tablet was dissolved into 50 ml RPMI1640 media to get 8.7 mmol/L 3TC stock solutions. The stock solutions were filtered for sterilization and diluted to the proper concentration before use. Nevirapine (NVP), containing 200 mg per tablet, was produced by Shanghai Disainuo biological medical company with authorization number H20020580. NVP stock solutions (15.0 mmol/L) were prepared and diluted to the proper concentrations with RPMI1640 media before use.

Generation of 3TC-resistant and NVP-resistant HIV-1 by *in vitro* passage

To generate 3TC-resistant virus, CNHN24 virus was used to infect MT-4 cells and selected with serial increasing concentrations of 3TC. MT-4 cells (5×10^4/ml) were infected with CNHN24 virus at 100 TCLD50 at an initial 3TC concentration of 0.008 μmol/L. Viral replication was monitored by observation for any cytopathic effect (CPE) present in the cultures. When CPE was seen, the viral supernatants were filtered and frozen at –80°C for analysis. Virus was serially passaged by using one aliquot of viral supernatant from the preceding passage to infect fresh MT-4 cells in the presence of increasing concentrations of 3TC, leading to the generation of viral stocks having increased resistance to 3TC. The drug concentrations used in the selection protocol varied, depending on the level of viral replication present in the preceding passage. Typically, virus from the preceding passage was used to infect fresh MT-4 cells in the subsequent passage at three different drug concentrations. The cultures were monitored for viral replication, and supernatants from successfully infected cultures grown in the presence of the highest drug concentration were used to infect fresh MT-4 cells in the following passage.

To generate NVP-resistant virus, MT-4 cells (5×10^4 /ml) were infected with CNHN24 virus at 100 tissue culture lethal dose 50 ($TCLD_{50}$) with NVP at an initial concentration of 1 mmol/L. The cells infected by virus were cultured with NVP for 8 days, washed, and then cultured in the presence of NVP at the concentration of 1 mmol/L until CPE were found.

Real-time RT-PCR

Viral RNA from wild-type and mutant stocks was quantified using real-time RT-PCR with an Applied Biosystems Prism 7300 sequence detection system (ABI). The supernatant was harvested at set time points and HIV-1 RNA was purified by use of the QIAmp viral RNA kit (Qiagen, Germany). Quantities of viral RNAs were determined by quantitative real-time RT-PCR. For these experiments, primers listed in Table 1 were used to amplify the region of HIV-1 reverse transcriptase (RT) containing the 184th amino acid or 181st amino acid. TaqMan minor groove binder (MGB) probes with reporting fluorescent FAM label at the 5′ end and a non-fluorescent quencher at the 3′ end were used to test copies of RNA of every virus in real-time RT-PCR. The reaction was carried out in MicroAmp optical 96-well reaction plates in a volume of 50 μl. The reactions contained 1 μl each of the two primers (concentration of primer and probe stock solutions, 20 μmol/L), 25 μl of One-step RT-PCR Master mix, 0.5 μl of RNase inhibitor, and 2 μl of viral RNA extract, 1 μl of MGB probe testing wild type virus or virus with mutation. The cycling parameters used for these experiments were as follows: 48°C for 30 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The probes used in the experiments were designed to distinguish one nuclei acid difference and their efficiencies had been tested.

Table 1. Sequences of primers and probes used in real-time RT-PCR

Sites	Categories	Names	Sequences
184	Upstream	FP1	AAAGGATCACCAGCAATATTCCA
	Downstream	RP1	TTTGTTCTATGCTGCCCTATTTCTAA
	Probe for wild type	184wt*	FTCAATAC<u>ATG</u>GATGATTTGTATGTAP
	Probe for mutation	184mu*	FCAATAC<u>ATA</u>GATGATTTATATATGTAGGP
181	Upstream	FP2	CAGCAATATTCCAATGTAGCATGAC
	Downstream	RP2	TTTGTTCTATGCTGCCCTATTTCTAA
	Probe for wild type	181wt†	FCCAGACATAGTTATC<u>TAT</u>CAP
	Probe for mutation	181mu†	FCCAGACATAGTTATC<u>ATT</u>CAP

Probes listed here are TaqMan MGB probes, 5′ end was labeled with reporter flurescent FAM, 3′ end was non-flurescent quencher. 184wt* was used to test wild type virus, 184mu* was used to test virus with M184I mutation; 181wt† was used to test wild type virus, 181mu† was used to test virus with Y181I mutation.

Calculation of RNA copies of virus in competition assay

In order to quantify RNA copies of each virus, standard curves were set up. For those experiments, wild type, 3TC-resistant virus and NVP-resistant virus were used to infect MT-4 cells separately. The supernatants were harvested 48 hours after inoculation. Viral RNA was purified by use of the QIAmp viral RNA kit. RNA copies were tested with NucliSens Easy Q assay (Biomerieux Company, NASBA). To establish a standard curve for HIV-1 RNA, we used serial dilutions, starting at a determined concentration, in real-time RT-PCR using methods shown above. The number of cycles required to reach threshold fluorescence (*Ct*) was determined. The Ct values and RNA copies obtained from serial dilutions of viral RNA were plotted to create a standard curves (data not shown). The stand curves were used to calculate the RNA copies of the mutant and wild-type HIV-1 RNAs that were used for the competition experiments. All of samples including those for graphing of the standard curve and testing were tested in the same conditions.

Competitive replication assay of drug-resistant strains and wild type strains

The fitness of HIV-1 strains containing the 3TC-resistant mutant or the NVP-resistant mutation relative to the wild-type CNHN24 were determined by competitive replication assay. MT-4 cells (5×10^6) were infected with equivalent p24 concentrations at the level of 10 ng/ml of each virus. After 2 hours incubation with virus, the cells were washed twice in phosphate-buffered saline to remove residual input virus that remained in the

supernatant. Cells were then cultured in media with 0.5 μmol/L drugs (3TC or NVP) or no drugs. After being cultured for 6 days, the supernatant was harvested and 200 μl of supernatant was transferred to a fresh culture of 5×10^6 MT-4 cells, in 10 ml of medium. This time point was set as the starting point for the competition experiment. Viral RNA was purified from the supernatant obtained at this time point to determine the ratio of wild-type and mutant viruses. To determine the change in this ratio over time, supernatants were harvested every 6 days and 200 μl of supernatant was passaged. HIV-1 primers, designed for the two regions of the RT where the mutations resided, were used to amplify a partial HIV-1 RT gene. One set, FP1 and RP1, amplified the region that contained amino acids 184 of RT. The other set, FP2 and RP2, amplified the region that contained amino acids 181 of RT. A one-step RT-PCR (performed in duplicate) was used to amplify these templates. For detection of the HIV-1 PCR products, TaqMan MGB probes specific for wild-type and mutant sequences (one mutant and one wild-type probe was included in each separate reaction) were designed to span regions that were specific for each mutation: 184mu and 184wt for residue 184, 181mu and 181wt for residue 181. Every RNA sample was tested in two separate wells for RNA of the wild type and of the mutant. All MGB probes were labeled with the reporter dye FAM (6-carboxyfluorescein) at their 5′ ends, and all probes had a nonfluorescent quencher at their 3′ ends. Post run manipulations of data were performed according to the manufacturer's instructions and as described above to calculate RNA copies with standard curve for the two viral species in each sample.

RESULTS

Selection of 3TC resistant variants

A 3TC resistant strain was selected *in vitro* by passaging wild type virus in the presence of increasing concentrations of 3TC (Table 2). The virus was initially propagated in the presence of 0.008 μmol/L 3TC (P1), and during the course of the selection procedure, the drug concentration was increased to 4.096 μmol/L (P10). At each passage the supernatant containing the virus was harvested and titrated for its infectivity and the sensitivity of the virus to 3TC was determined by the MTT assay (Table 2). The inhibition concentration 50% (IC_{50}) of 3TC against wild type CNHN24 and P10 were 0.018 and 2048 μmol/L, respectively. The fold difference in the IC_{50} was 110 000. The amino acid sequence of the RT-encoding region of virus at each passage was determined by using the nest-PCR method.[7] At passage 7 (in the presence of 0.512 μmol/L 3TC), a mutation at codon 184 from Met to Ile was first identified.

Selection of NVP resistant variants

To select variants resistant to NVP, MT-4 cells were infected with CNHN24 and cultured in the media containing NVP at a concentration of 1 mmol/L (Table 3). Cells were cultured for 8 days before being washed and

Table 2. Induction of resistant strain with M184I and genotype/phenotype identification

Passages	Days of culture	Concentration (μmol/L)	IC_{50} (μmol/L)	184 Codon	184 Amino acid	Fold of IC_{50}*
0	—	—	0.018†	ATG	M§	—
1	5	0.008	ND‡	—	ND	—
2	5	0.016	ND	—	ND	—
3	5	0.032	0.026	ATG	M	1.4
4	5	0.064	ND	—	ND	—
5	6	0.128	0.15	—	ND	8.3
6	6	0.256	0.15	ATG	M	8.3
7	6	0.512	>2048	ATA	I‖	>113 777.8
8	6	1.024	ND	—	ND	—
9	7	2.048	ND	ATA	I	—
10	7	4.096	>2048	ATA	I	>113 777.8

*Fold of resistance relative to wild type. †IC_{50} for 3TC of wild type. ‡Not done. §Methionine Met. ‖Isoleucine Ile.

Table 3. Induced resistant strain with Y181I and genotype/phenotype identification

Passages	Days of culture	Concentration (μmol/L)	IC_{50} (μmol/L)	181 Codon	181 Amino acid	Fold of IC_{50}
0	—	—	0.027	TAT	Y	1
1	8	1000	ND	ND	—	—
2	8	1000	ND	ND	—	—
3	5	1000	ND	ND	—	—
4	5	1000	>1000	ATT	I	>37 000

passaged in media with NVP at the concentration of 1 mmol/L. Significant CPE was found after 4 passages. The viruses in the supernatant were recovered and the proviral DNA sequence of the RT region was amplified and sequenced; a Y181I mutation was found. The infectivity and sensitivity of the variants to NVP were determined. The IC_{50} of NVP against wild type CNHN24 and the variants were 0.027 and >1000 μmol/L, respectively. The fold difference in the IC_{50} was more than 37 000.

Competitive fitness of drug-resistant HIV-1 strains

We observed that in the presence of 3TC, virus containing the M184I mutation displayed an increased level of fitness relative to the wild type virus (Figure 1). After 4 passages in the competitive culturing system in the presence of 3TC, wild type virus was nearly replaced by the drug-resistant strain (the ratio of drug-resistant variant increased to 98.8%). The same phenomenon was observed for the virus containing the Y181I mutation; the Y181I variants outgrow wild type virus after 4 passages. These experiments revealed that in the presence of drugs, virus carrying the M184I mutation and the Y181I mutation are both fitter than wild type virus.

The competitive replication assay in the absence of drugs revealed that virus containing the M184I mutation or the Y181I mutation gave a different replication profile (Figure 2). The ratio of the M184I variant decreased during passages in vitro compared to the wild type virus in the absence of 3TC. After 4 passages, the ratio of the M184I variant fell to 5% while the wild type virus increased to 95%. However, the Y181I mutant grew faster than the wild type virus even in the absence of NVP; the ratio of the Y181I variant increased to 75%, suggesting that the Y181I variant was fitter than the wild type virus.

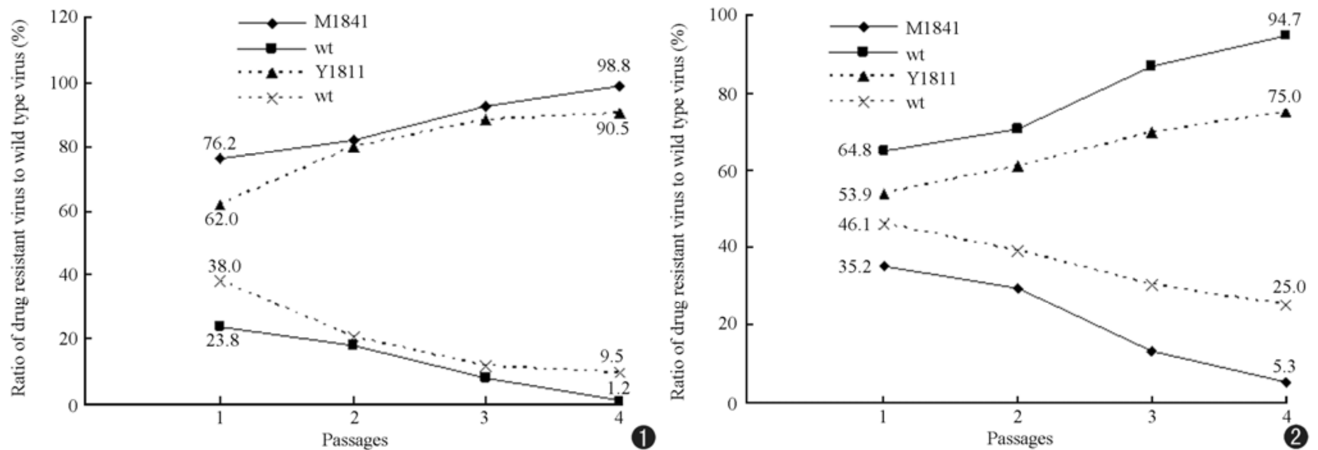

Figure 1. Percentages of drug resistant virus and wild type virus under drug pressure plotted over time. Viruses containing M184I or Y181I were co-cultured separately with wild type virus *in vitro* with accordingly drug at the concentration of 0.5 μmol/L. The viruses were passaged 4 times and analyzed using real-time RT-PCR.

Figure 2. Percentages of drug resistant virus and wild type virus without drug pressure plotted over time. Viruses containing M184I or Y181I were co-cultured separately with wild type virus *in vitro* in the absence of drugs. The viruses were passaged 4 times and analyzed using real-time RT-PCR.

DISCUSSION

In the present study, we selected HIV-1 variants resistant to 3TC by exposing CNHN24 strain to increasing concentrations of the drug. HIV-1, propagated in the presence of 0.512 μmol/L 3TC, proved to be highly resistant to 3TC (Table 2). We analyzed the nucleotide sequence of the RT-encoding region at passage 3, 6, 7, 9, 10 and first identified a M184I mutation at passage 7, which was considered to be responsible for the observed viral resistance to 3TC.[8,9] NVP-resistant virus was selected in different way. Wild type virus was directly exposed to high levels of NVP (30 000-fold of the IC_{50} for drug-sensitive virus), and passaged with drugs 4 times until CPE was observed. Genotyping analysis showed that there was a mutation in the code for the 181st amino acid of RT (from TAT to ATT), which caused an amino acid change from Y to I.

Resistance is conferred by mutations in the viral genome that result from the combination of a lack of proofreading activity of RT, and high viral replication rate.[10,11] The loss of antiviral activity of anti-HIV-1 agents by the selection of a single mutation represents a low genetic barrier to resistance. That may be the reason why 3TC-resistant strains (G to A) could be easily selected with increasing drug pressure.[12] Compared to 3TC, NVP was shown to have a higher genetic barrier to the development of resistance since two mutations were needed to confer significant resistance (TA to AT) and this may be the reason why NVP-resistant virus can not be selected with the same strategy as selecting 3TC-resistant virus.

Some reports showed that HIV-1 M184I emerged first, and shortly thereafter HIV-1 M184V becomes dominant in patients receiving 3TC.[13-15] The same phenomenon was observed *in vitro*.[16] Therefore, expecting to detect the M184V mutation, we continued to select the M184I variant for an additional 3 passages in the presence of up to 4.096 μmol/L 3TC; however, M184V failed to emerge even at passage 10. So, it may not be easy to transform from M184I to M184V for Chinese HIV-1 strains. Considering the limitations in our finding, further study *in vivo* is needed.

In this study, we demonstrated the utility of a quantitative real-time RT-PCR-based competition assay for evaluating the relative fitness of drug-resistant HIV-1 strains. This system addressed many of the issues that have limited previous studies pertaining to this topic, as follows. (1) By utilizing isogenic strains differing by as little as a single nucleotide, we eliminated the potential confounding effects on viral fitness of sequence variables outside the mutation of interest,[14,17] i.e., in other genes, such as *env*. However, the use of a single genetic background does not allow for the quantification of the effect of nonresistance RT polymorphisms on the fitness of nevirapine-resistant mutants. (2) This method provided a level of sensitivity that allowed for measurable differences in the relative fitness of variant strains to be determined, which could not be done with a growth assay or P24-analysis-based method.[18] (3) This assay more closely resembled *in vivo* conditions in which mutant and wild-type strains are maintained in the same environment. In some previous studies, culture of drug-resistant virus with drug-sensitive virus in parallel was used to test viral replication capacity, those results were relatively unconvincing because viruses could not compete in a same replication environment.[19,20]

Real-time RT-PCR is a sensitive and specific method for quantifying viral RNA. In the present study, low level virus (the P24 concentration of each virus was 10 ng/ml) was used to infect cells, so that the mismatch between probes and templates can be minimized, and low level of recombination between wild type virus and drug-resistant

virus will occur. In the experiments, low level drugs were adopted in the competitive replication assay so that wild type virus could not be absolutely inhibited and could compete with drug-resistant virus, although the drug resistant variants used in experiments could endure higher drug levels.

Previous studies had shown that a mutation at the 184th position will lower the replication capacity of virus to 3%–10%,[21] or even 48%–57%,[22] compared to wild type virus. When the selection pressure of drugs disappeared, 3TC-resistant virus will be released from the pressure of drugs with the mutation disappearing quickly. The replication capacity of the NVP-resistant virus was different from 3TC-resistant strains; the replication capacity of NVP-resistant virus with a 181st position mutation is higher than that of wild type virus even without drugs. This phenomenon is contrary to the normal opinion that the replication capacity of the wild type is always higher than mutant virus. In Collins′ study, NVP-resistant virus containing a Y181C mutation, but not Y181I, was reported to have lower replication fitness comparing to wild type virus without drug pressure.[23] It was considered that two reasons may contribute to the explanation. First, a Chinese isolate was used, while NL4-3 virus was used in Collins′s paper. Second, the mutations causing NVP-resistance are different. An amino acid difference at the 181 site (C or I) may change the replication fitness of the virus.

Our work showed that virus containing Y181I mutations has higher replication capacity than the wild type strain; the results are consistent with many reports. Many reports have showed that virus containing mutations at the 181st site have a higher replication capacity than other drug-resistant virus[24] and that no recovery mutations were found during passages in environments without drugs.[25] Research *in vivo* also showed that in a patient who had quit therapy with NVP for more than 20 months, this mutation in virus still could be found.[26] Since HIV virus containing Y181I mutaion is fitter than wild type virus, then why did it not become the “wild type virus”? It may be because that no enough virus is present for a quasispecies to become a dominant quasispecies.[27] In addition, it may be caused by the interactions between quasispecies[28] or the appearence frequencies of different strains,[29] etc.

In this study, we found that variants containing M184I or Y181I mutations have different replication capacity comparing to wild type virus in the absence of drugs. These results are consistent with what we found *in vivo*. In patients taking highly active antiretroviral therapy, virus with NRTIs related mutations will disappear shortly after the patient adherence was improved or therapy was interrupted. However, virus with NNRTIs mutations will remain for a long time. It was hypothesized that the decreased replication capacity of virus caused by NRTIs-resistant mutations, for example M184I, may result in the disappearance of drug-resistant virus when the selection pressure *in vivo* is removed. For virus containing some main NNRTIs mutations, the opposite may be true. The replication capacity of those NNRTIs-resistant virus is not lower than wild type virus, some of them are even higher than wild type virus, as the result, NNRTIs-resistant variants may replicate *in vivo* for a long time.

An understanding of the mechanisms of development of resistance is essential in order to maximize the clinical benefit of new antiretroviral agents.[30] *In vitro* experiments can be a useful indicator of the replication capacity of drug-resistant virus, however, the experimental conditions *in vitro* may affect the replication capacity of drug-resistant virus; accordingly the predictive value of *in vitro* data for the fitness of virus containing drug-resistant mutations *in vivo* may be limited, so studies *in vivo* to prove the results will be required.

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