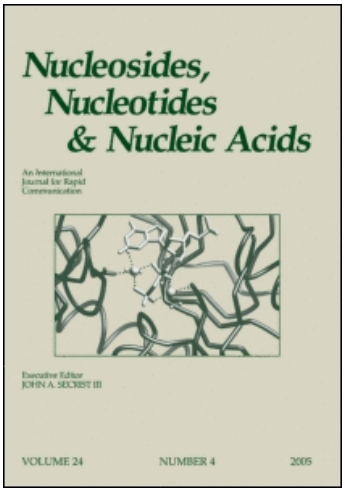

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IN VITRO SELECTION AND CHARACTERIZATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 RESISTANT TO ZIDOVUDINE AND TENOFOVIR

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□ *In this study, we undertook to generate HIV-1 resistance to PMPA by in vitro passage and to characterize the cross-resistance patterns and RT mutations in the generated resistant virus. The HIV-1 A102-resistant to AZT was serially passaged for 4 months in the presence of increasing concentrations of PMPA up to maximum of 40 μM on the fresh MT-2 cells. After 25 passages, HIV-1 developed decreased sensitivity to PMPA after long-term in vitro exposure. Selected HIV-1 mutants were characterized by decreased susceptibility to PMPA (4-fold). This decrease could be related to PMPA resistant caused by an amino acid change associated with a V148M substitution. From these results, additional studies will be needed to determine whether a similar mutation in HIV RT develops in patients receiving PMPA or its orally bioavailable prodrug, tenofovir dipivoxil fumarate.*

Keywords AZT; HIV-1 resistant; tenofovir

The advent of drug-resistant mutants of human immunodeficiency virus type 1 (HIV-1) has emerged as an important obstacle in antiviral therapy for AIDS. Therefore, highly active antiretroviral therapy has been limited by the emergence of multidrug-resistant HIV-1.[1,2] Tenofovir [*R*-9-(2-phosphonomethoxypropyl) adenine (PMPA)] is a nucleotide analogue inhibitor of the HIV-1 reverse transcriptase (rt).[3–5]

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The purpose of this study was to determine whether HIV-1 resistance to PMPA could be generated by in vitro selection and if so, to determine which mutations in rt were responsible. Therefore, we performed generation of HIV-1 resistance to PMPA by in vitro passage and to characterization of cross-resistance patterns and RT mutations in the generated resistant virus.

MT-2 cells (10^5 cells/ml) were infected for 2 hours to $TCID_{50}$ of HIV-1 A012 (NIH AIDS Research & Reference Reagent Program, Germantown, MD, USA), the cell was washed to remove residual virus and resuspended in 2 ml fresh media in the presence or absence of 0.4 μM PMPA of 6-well plate (Falcon, Becton Dickinson and Company, Germantown, MD, USA) at 37°C in humidified air supplemented with 5% CO_2. After 5 days, the culture supernatant was harvested and filtered through a 0.45 μm filter. Subsequent passages were performed in the same manner except that 100 μl of the culture supernatant from the previous passage were used to infect the fresh MT-2 cells. The time for each passage was 5 days. Aliquots of supernatant were also periodically frozen at -70°C. The concentration of PMPA in the cultures was increased over 25 passages from 0.4 to 40 μM.

The HIV-1 A102 resistant to AZT was serially passaged for 4 months in the presence of increasing concentrations of PMPA up to maximum of 40 μM on the fresh MT-2 cells. After 25 passages, HIV-1 developed decreased sensitivity to PMPA after long-term in vitro exposure and the presence of infectious HIV-1 by assessing the CPE (cytopathic effect) and was monitored by p24 release assay (Vironostika, BioMerieux Co., the Netherlands). Virus production was assessed by p24 antigen production, and the IC_{50} calculated by linear regression.

The susceptibility assay for CUK-2 to drugs was performed with infection of virus (moi 0.1) into MT-4 cell. Testing of viral sensitivities to drugs in MT-4 cells by P24 release assay in triplicate. The MT-4 cells were pre-exposed to virus for 2 hours, washed, and cultured in the presence of various concentrations of drugs in triplicate for 5 days. The same amount of the three viruses added to the drug susceptibility assay was determined by P24 assay. Selected HIV-1 mutants were characterized by decreased susceptibility to PMPA (4-fold). This decrease could be related resistant to PMPA resistance caused by an amino acid change. This data are shown in Table 1. On the other hand, in the level of AZT resistance, NL4-3 wild type, HIV-1 A102, and CUK-2, HIV-1 A102 selected with PMPA showed IC_{50} values of 0.06, 30.12, and 35.64, respectively. The PMPA selected virus maintained the same level of AZT resistance as the original AZT-resistant parental isolate HIV-1 A102 when compared to NL4-3 wild type (both viruses are approximately 500- to 600-fold resistant).

A 931-bp fragment of the HIV-1 *pol* gene was amplified by PCR using the primer pairs 5′GGGGGAATTGGAGGTTTTATCAAAG 3′ and 5′TTCTGAATGTCATTGACAGTCCAGC 3′ This segment includes almost all

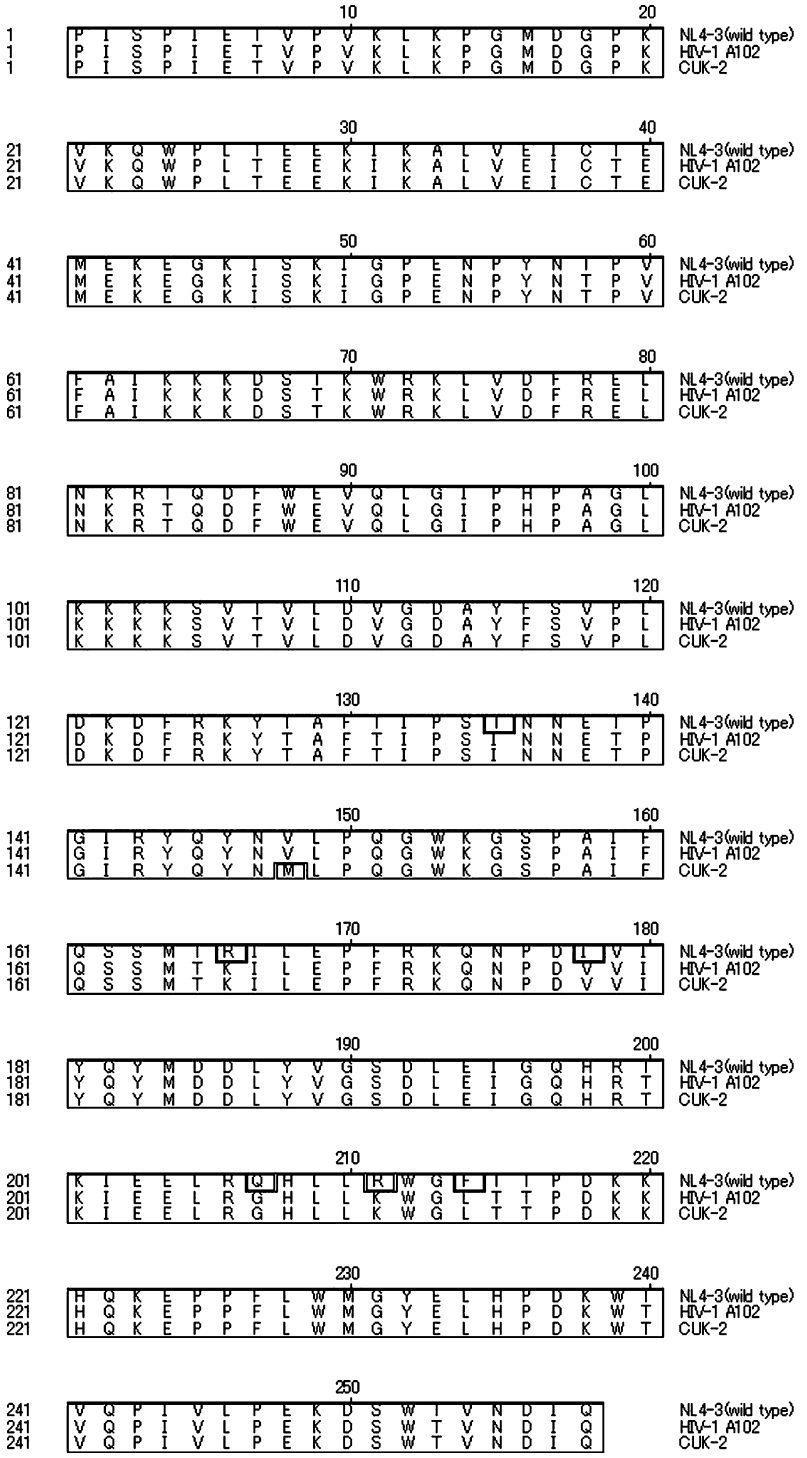

FIGURE 1 Comparison of the amino acid sequences of RT region (1–258) of HIV-1 PMPA resistant strain, CUK-2. Small boxes indicate amino acids unidentical to the respective amino acids of three HIV-1 virus strains.

TABLE 1 Antiviral susceptibilities of the PMPA resistant HIV-1 strain CUK-2 measured by p24 release assay

	IC_{50} (μM)	
Virus	AZT	PMPA
Wild type (NL4-3)	0.06 (±0.02)	2.82 (±0.69)
HIV-1 A102 (AZT-R)	26.81 (±4.10)	5.21 (±1.17)
HIV-1 A102[a]	30.12 (±3.48)	6.42 (±1.81)
CUK-2[b]	35.64 (±4.78)	28.03 (±5.30)

[a]HIV-1 A102 after 25 passaged in medium without PMPA.
[b]HIV-1 A102 after 25 passaged in medium with PMPA.
(±) standard deviation of 3 times experiments.

the HIV mutations that have been previously reported.[8] Five clones that have PMPA-resistant HIV-1 were analyzed nucleic acid and translated amino acid sequences. Four of these five clones were found to be V148M mutation. Two mutations in the *pol* gene (GenBank accession no. EF137864) of PMPA resistant HIV-1 were identified as $_{277}$ GGA-> GGT and $_{442}$ GTG->ATG in RT nucleic acid sequences. This suggests that $_{442}$ GTG->ATG is related to the V148M substitution and $_{277}$ GGA-> GGT is silent mutation. Consequently, only one mutational change V148M in RT region was identified although most resistant HIV-1 strains against tenofovir currently reported has been known as K65R. We compared the amino acid sequences of PMPA resistant HIV-1 strain, CUK-2 with NL4-3 (wild type) and AZT resistant HIV-1 strain A102 (Figure 1).

Based on these results, additional investigation will be requested for the study on mutation in RT of HIV-1 in patients receiving PMPA or its orally bioavailable prodrug, tenofovir dipivoxil fumarate.

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