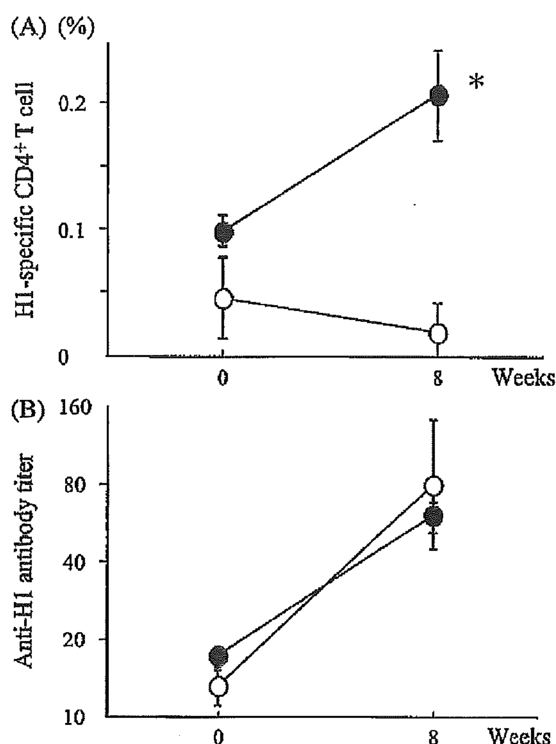

FIGURE 3. H1-specific CD4+ T cells and anti-H1 antibody responses at week 8 after vaccination in influenza A/H1N1–infected patients. Five vaccinated individuals were infected with influenza A/H1N1. A, Percentage of H1-specific CD4+ T-cell responses in infected (○; n = 4) and noninfected (●; n = 119) individuals. *H1-specific CD4+ T cells responded better to influenza A/H1N1 in noninfected patients than in infected patients ($P < 0.05$). One sample of 5 influenza A/H1N1–infected individuals was not examined because the sample was not among the first 10 samples per day as stated in the text. B, Anti-H1 antibody titers in infected (○; n = 5) and noninfected (●; n = 249) individuals. The anti-H1 antibody response at week 8 was similar in both groups. Data are mean ± SEM.

baseline antibody-negative HIV-1–infected patients, the antibody responses to the single-shot vaccination were less effective than those in healthy patients. In contrast, however, in baseline antibody-positive HIV-1–infected patients, the antibody responses were similar or more effective than those in the healthy controls and the titers exceeded >40 U in most cases, irrespective of CD4 count. Previous studies demonstrated that an antibody titer >40 U could be used as an index of vaccine protection.[12,25] In our study, the antibody titer was <40 U in most patients who became infected with influenza. Considered together, these results suggest that the antibody response may support the clinical efficacy of influenza vaccination. Kroon et al[8] reported that postvaccination antibody titers were higher in previously vaccinated HIV-1–infected patients than in nonvaccinated patients, although the difference was not significant. In the present study, the antibody titers showed a better response in individuals positive at baseline for anti-HA antibody than in those negative for the antibody. Furthermore, the response was well sustained, irrespective of CD4 count. Thus, it is conceivable that annual vaccination is specifically important for all HIV-1–infected patients. Sustainability of the antibody titer raised by the vaccination is to be followed in a future study.

In the immunologic part of our study, we examined antibody responses and specific CD4 T cells. The antibody response was almost the same as that reported previously[8,9]; the response correlated with the CD4 count. In contrast, specific CD4 T cells were much more influenced by HIV VL than by CD4 count.[1,8–15] Therefore, the specific CD4 T cells were higher in patients treated with HAART than in those untreated. This result indicates that HAART improves HA-specific CD4 T cells like in other infections,[21] or, in other words, the heightened cellular response to the influenza vaccine suggests functional reconstitution of the immune system after HAART.

Our data indicate that the specific CD4 T-cell responses may be related to HIV VL. The specific CD4 T-cell response needs antigen presentation by dendritic cells.[26] HIV-1 infection impairs the function of antigen presentation of dendritic cells.[27] Therefore, specific CD4 T-cell responses may be profoundly decreased in patients with a high HIV VL.

It is interesting to note that the percentage of H1-specific CD4 T cells at week 8 was significantly lower in influenza A/H1N1–infected patients. It is conceivable that the response of HA-specific CD4 T cells at week 8 can predict the efficacy of influenza vaccine. Influenza-specific CD4 T cells provide help (as Th cells) to B cells for the production of antibody to influenza HA and neuraminidase[28,29] and also promote the generation of virus-specific CD8+ cytotoxic T lymphocytes (CTLs).[26,30–33] Therefore, the specific CD4 T cell must have a protective role. This concept would be more reliable if we had analyzed H3-specific CD4 T cells rather than H1-specific CD4 T cells, because influenza A/H3N2 was the predominant subtype in this season. Further studies are necessary to elucidate this point.

Our study was designed as a prospective but nonrandomized study, because influenza vaccine has been already recommended for HIV-1–infected patients.[7] Practically, the number of nonvaccinated patients who did not participate in our study was higher than that of vaccinated patients (13% of nonvaccinated patients vs. 4.5% of vaccinated patients), and the violation rate of the study protocol was higher in nonvaccinated patients than in vaccinated patients (24.1% vs. 17.4%). Thus, 262 (78.9%) of 332 vaccinated patients and 66 (66%) of 100 nonvaccinated patients were analyzed in this study. Although a relatively high proportion of patients failed to complete the protocol, the main reason for the drop out may have been the lack of incentives and the need to visit our clinic on a fixed date for blood sampling. The vaccinated and nonvaccinated groups were well balanced in terms of baseline characteristics, however. Finally, we believe that the selection bias of participants, if any, is negligible.

In conclusion, our prospective study in a large population demonstrated that influenza vaccine provides protection of HIV-1–infected patients. In baseline antibody-negative patients, the antibody responses to the vaccination were significant in those patients with a CD4 count >200 cells/μL compared with those with a CD4 count <200 cells/μL. In contrast, in baseline antibody-positive patients, good antibody responses were observed, irrespective of CD4 counts. Annual vaccination of

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HIV-1–infected patients is thus recommended. Therapy with HAART improves the cellular immune response to influenza HA.

ACKNOWLEDGMENTS

The authors are indebted to Dr. N. Sugaya from the Keiyu Hospital for helpful suggestions regarding this study and to Drs. T. Gotanda and Y. Suzuki from the Kitasato Institute Research Center for Biologicals for kindly providing purified HA obtained from influenza virus A/New Caledonia/20/99. The authors thank Dr. N. Ishizuka, International Medical Center of Japan, for special advice on interpretation of the statistical analyses. We also thank F. Negishi and Y. Takahashi for sample stock, preparation, and technical support.

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APPENDIX

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BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. 20: 28–36 (2006)
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bmc.521

ORIGINAL RESEARCH

Simultaneous determination of six HIV protease inhibitors (amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir), the active metabolite of nelfinavir (M8) and non-nucleoside reverse transcriptase inhibitor (efavirenz) in human plasma by high-performance liquid chromatography

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Received 9 December 2004; revised 10 March 2005; accepted 15 March 2005

ABSTRACT: We report the development of a simple, economical and reliable chromatographic method for the simultaneous determination of six HIV protease inhibitors (PIs; amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir), the active metabolite of nelfinavir (M8) and the non-nucleoside reverse transcriptase inhibitor (NNRTI; efavirenz) in human plasma. After extraction from plasma with an ethyl acetate–acetonitrile mixture, the analytes were separated on a phenyl column with a gradient of acetonitrile and phosphate solutions, and detected at three ultraviolet wavelengths. Calibration curves were linear over the range 0.025–15 μg/mL for saquinavir and 0.05–15 μg/mL for the other analytes. The accuracies ranged from −6.9% to +7.6%, and the intra-assay and inter-assay precisions were <9.2 and <11.8%, respectively. Our method, covering most of the PIs and NNRTIs currently used, facilitates ready therapeutic drug monitoring in hospital laboratories. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: protease inhibitors; efavirenz; HIV; therapeutic drug monitoring; high-performance liquid chromatography

INTRODUCTION

Currently, more than 17 anti-human immunodeficiency virus (HIV) drugs have been approved and are in clinical use in industrialized countries. To obtain optimal antiviral efficacy and to prevent viral drug resistance, these drugs are administered to patients in combination regimens, which are referred to as highly active antiretroviral therapy (HAART). Current standard HAART regimens consist of one or two protease inhibitors (PIs) or one non-nucleoside reverse transcriptase inhibitor (NNRTI), together with two nucleoside reverse transcriptase inhibitors (NRTIs; Richman, 2001; British HIV Association Writing Committee, 2003; The Panel on Clinical Practices for Treatment of HIV Infection, 2004; Yeni *et al.*, 2004), but more complex regimens are often needed because of treatment failure. Since the introduction of HAART in the late 1990s, the short-term prognosis of HIV infection has dramatically improved (Palella *et al.*, 1998). However, a large degree of inter-patient variability is observed in the efficacy and toxicity of HAART and still remains to be resolved.

This variability is at least in part attributed to the pharmacokinetics of the drugs, especially PIs and NNRTIs (Barry *et al.*, 1998; Acosta *et al.*, 1999; Marzolini *et al.*, 2001). Cytochrome P450 (CYP), by which PIs and NNRTIs are extensively metabolized, and P-glycoprotein, which plays an important role in transportation of these drugs at tissue and cellular levels, have genetic polymorphisms, leading to the inter-patient variability in pharmacokinetics (Fellay *et al.*, 2002). Furthermore, PIs and NNRTIs are both potent CYP inducers and inhibitors, and as a result, complicated and unpredictable pharmacokinetic interactions with co-administered drugs frequently occur (Dresser *et al.*, 2000; Gerber, 2000). To overcome

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Abbreviations used: APV, amprenavir; CYP, cytochrome P450; EFV, efavirenz; HAART, highly active antiretroviral therapy; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; RTV, ritonavir; SQV, saquinavir; TDM, therapeutic drug monitoring.

Contract/grant sponsor: Ministry of Health, Labour and Welfare of Japan; Contract/grant number: H15-15100101.
Contract/grant sponsor: Japanese Foundation for AIDS Prevention.
Contract/grant sponsor: Organization of Pharmaceutical Safety and Research; Contract/grant number: 01-4.

Published online 22 June 2005

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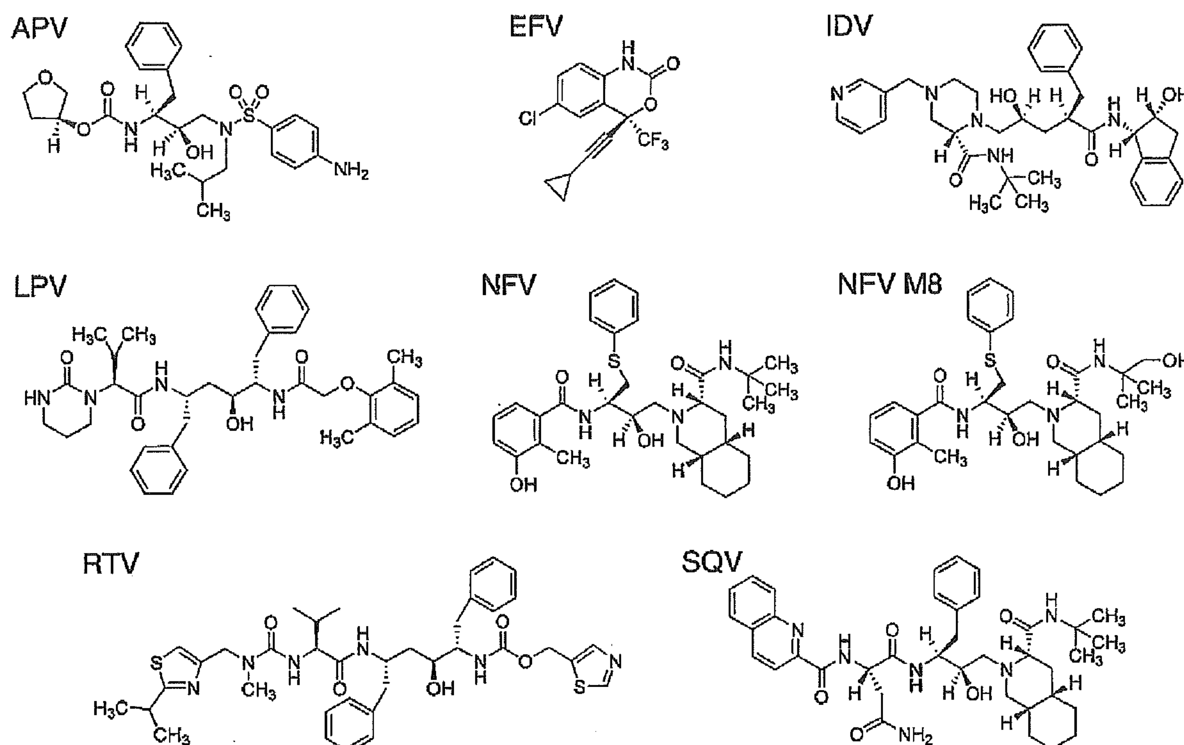

Figure 1. Chemical structures of APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV.

the pharmacokinetic variability, therapeutic drug monitoring (TDM) for PIs and NNRTIs has recently been proposed as a practical and potential tool by clinicians (Acosta *et al.*, 2002; Back *et al.*, 2002; Van Heeswijk, 2002). In addition, TDM may be useful in assessing adherence to treatment, which is another clinical problem in HAART (Hugen *et al.*, 2002).

For the purpose of routine TDM in hospital laboratories, a simple and reliable analytical method that can simultaneously determine plasma concentrations of most PIs and NNRTIs is highly desirable. In this article, we describe a novel chromatographic method for the simultaneous determination of the six widely used PIs [amprenavir (APV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV); Fig. 1] and a clinically important active metabolite of NFV, M8, together with efavirenz (EFV), which is the most frequently used NNRTI. Moreover, the usefulness of this method for TDM is discussed.

EXPERIMENTAL

Materials. NFV mesylate and its metabolite M8 were kindly provided by Japan Tobacco Inc. (Tokyo, Japan), IDV sulfate and EFV by Merck & Co. (Rahway, NJ, USA), RTV and LPV by Abbott Laboratories (Abbott Park, IL, USA), APV by Kissei Pharmaceutical Co. (Matsumoto City, Japan) and SQV mesylate by Roche Products (Welwyn Garden City, UK). The compounds tested for possible interference with the analytical method were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan), or were extracted from commercial products. Acetonitrile, methanol, distilled water (each of HPLC grade), disodium hydrogenphosphate (Na_2HPO_4), sodium dihydrogenphosphate (NaH_2PO_4) and sodium 1-hexanesulfonate were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Ethyl acetate (HPLC grade), *n*-hexane (HPLC grade), ammonium hydroxide and phosphoric acid were obtained from Wako Pure Chemical Industries.

Drug-free blank plasma was collected from eight healthy volunteers in our hospital. Clinical samples were obtained from 10 HIV-infected patients receiving PIs and/or EFV as part of a HAART regimen. The Ethics Committee for the Clinical Study in our hospital approved this study (no. 39) and all participants provided written informed consent. The blood was drawn into heparinized tubes, and plasma was separated by centrifugation at 3000 *g* for 10 min and stored at −30°C.

Separate stock solutions of the eight analytes were prepared by dissolving the compound in methanol to yield a concentration of 1000 μg/mL as free base. Each solution was combined and diluted in 50% (v/v) methanol to give a working solution containing all eight analytes at 100 μg/mL. The working solution was further diluted in blank plasma for the preparation of calibration standards and quality controls.

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Sample pretreatment. An aliquot of 400 μL plasma was transferred to a 5 mL glass tube and alkalinized with 400 μL of 0.1 M ammonium hydroxide adjusted to pH 10.5 with phosphoric acid. After vortexing briefly, 1.8 mL of an ethyl acetate–acetonitrile mixture (9:1, v/v), freshly prepared, was added to each tube. The tubes were vortexed vigorously at room temperature for 5 min and centrifuged at 2000 *g* at 4°C for 10 min. Then, 1.5 mL of the organic phase was transferred to a 2 mL tube and evaporated to dryness under a gentle stream of nitrogen at 40°C. Subsequently, the residue was reconstituted in 150 μL of solvent A (see 'Chromatography'). The resulting solution was washed with 1 mL *n*-hexane by vortexing for 10 s and centrifuging at 2000 *g* for 2 min. After eliminating the upper hexane layer, the non-hexane solution was filtrated through a 0.45 μm filter and transferred to an auto-sampler vial. Aliquots of 50 μL were injected into the HPLC unit.

Chromatography. Chromatography was performed using an integrated high-performance liquid chromatography (HPLC) unit, Integral 100Q (Applied Biosystems, Foster City, CA, USA), which consists of an automatic sample injector, a binary solvent delivery pump and a dual-wavelength ultraviolet detector. The analytical column was a Develosil Ph-UG-3 column (150 × 2.0 mm, 3 μm particle size; Nomura Chemical Co., Seto City, Japan) protected by a Develosil Ph-UG-S precolumn (10 × 1.5 mm; Nomura Chemical Co.). The temperature was maintained at 40°C using a Shodex column heater (Showa Denko Co., Tokyo, Japan). Analytical runs were processed by the Turbochrom software (version 3.01; Applied Biosystems).

The mobile phase consisted of a gradient of solvents A and B. Solvent A was a mixture of 34% (v/v) acetonitrile and 66% (v/v) 25 mM NaH_2PO_4 containing 6 mM sodium 1-hexanesulfonate and exactly adjusted to pH 5.1 with 25 mM Na_2HPO_4. Solvent B was composed of 64% (v/v) acetonitrile and 36% (v/v) 25 mM NaH_2PO_4 containing 6 mM sodium hexanesulfonate and adjusted to pH 5.3. Over the first 14.6 min of the run, 100% solvent A was delivered constantly, and then the proportion of solvent B was increased linearly from 0% to 30% over 2.0 min. From time 16.6 to 35.5 min, a mixture of 70% solvent A and 30% solvent B was used in an isocratic mode. The column was then rinsed with 100% solvent B for 4.5 min and semi-equilibrated with 100% solvent A for 6.8 min. The flow rate was maintained at 200 μL/min. Prior to use, solvents were passed through a 0.45 μm filter and degassed by helium sparging.

Absorbance was monitored at a dual wavelength: channel 1 was set at a wavelength of 212 nm through the run. For channel 2, the wavelength was set at 266 nm for the first 18.3 min, then switched to 239 nm automatically and switched back to 266 nm at the end of the run. APV, SQV and the other six analytes were detected at 266, 239 and 212 nm, respectively.

Method validation. The calibration was established over the range of 0.025–15 μg/mL for SQV and 0.05–15 μg/mL for the other analytes using seven spiked plasma samples. The calibration curve for each analyte was constructed by least-squares linear regression of the observed peak area vs the spiked concentration. Unknown concentrations of quality controls and patient samples were calculated from the linear regression equation of daily calibration curve for each analyte. The data analyses were performed using Turbochrom software (version 3.01; Applied Biosystems) on a Windows personal computer.

The accuracy and precision of the method were determined by assaying plasma samples spiked with the analytes at three different concentrations of 0.3, 2.1 and 12 μg/mL. Accuracy was defined as the percentage of deviation from the nominal concentration. Intra-assay precision was calculated as the relative standard deviation from six replicate quality controls within a single assay. Inter-assay precision was estimated from the analyses of quality controls on five separate days. The lower limits of quantitation (LLQs) were determined with plasma samples spiked with decreasing concentrations of the analytes (0.0125, 0.025, 0.05 and 0.1 μg/mL). The LLQ was defined as the lowest concentration for which the percentage deviation from the nominal concentration and the relative standard deviation were both less than 20%. The LLQ values were used for the lowest concentration of daily calibration curves. The upper limit of quantitation (ULQ) was arbitrarily determined as 15 μg/mL for each analyte.

The efficiency of the extraction procedure was determined with plasma samples spiked at three different concentrations of 0.3, 2.1 and 12 μg/mL in triplicate. The average recovery of each analyte was calculated by comparing the observed peak areas of the processed samples with those of non-processed standard solutions in mobile phase (solvent A). The stability of the analytes in plasma samples was investigated for three different conditions. Plasma was spiked with two concentrations of the analytes (0.3 and 12 μg/mL) and subsequently kept at 60°C for 60 min, 4°C for 7 days and −30°C for 60 days including three freeze–thaw cycles. These samples were analyzed together with freshly prepared samples and the ratios of the observed concentrations were calculated.

RESULTS

Representative chromatograms of plasma spiked with 1.8 μg/mL of each of the six PIs, NFV M8 and EFV are shown in Fig. 2. IDV, NFV M8, EFV, RTV, LPV and NFV were detected at a wavelength of 212 nm [Fig. 2(A)], based on their ultraviolet absorption spectra (data not shown). APV and SQV were monitored at 266 and 239 nm, respectively [Fig. 2(B)], because interfering endogenous peaks were observed near the peaks of these analytes at 212 nm. The retention times were 14.4, 16.2, 21.8, 23.7, 25.8, 30.0, 31.4 and 33.0 min for IDV, APV, NFV M8, EFV, SQV, RTV, LPV and NFV, respectively.

Blank plasma samples from eight healthy individuals showed no endogenous peaks interfering with any analyte. The typical chromatograms are presented in Fig. 3. A total of 35 drugs, which are frequently co-administered to HIV-infected patients, were also examined for possible analytical interference (Table 1). All the drugs but ketoconazole were eluted at retention times of <10 min or were not detected with the method. Ketoconazole had a retention time of 20.2 min,

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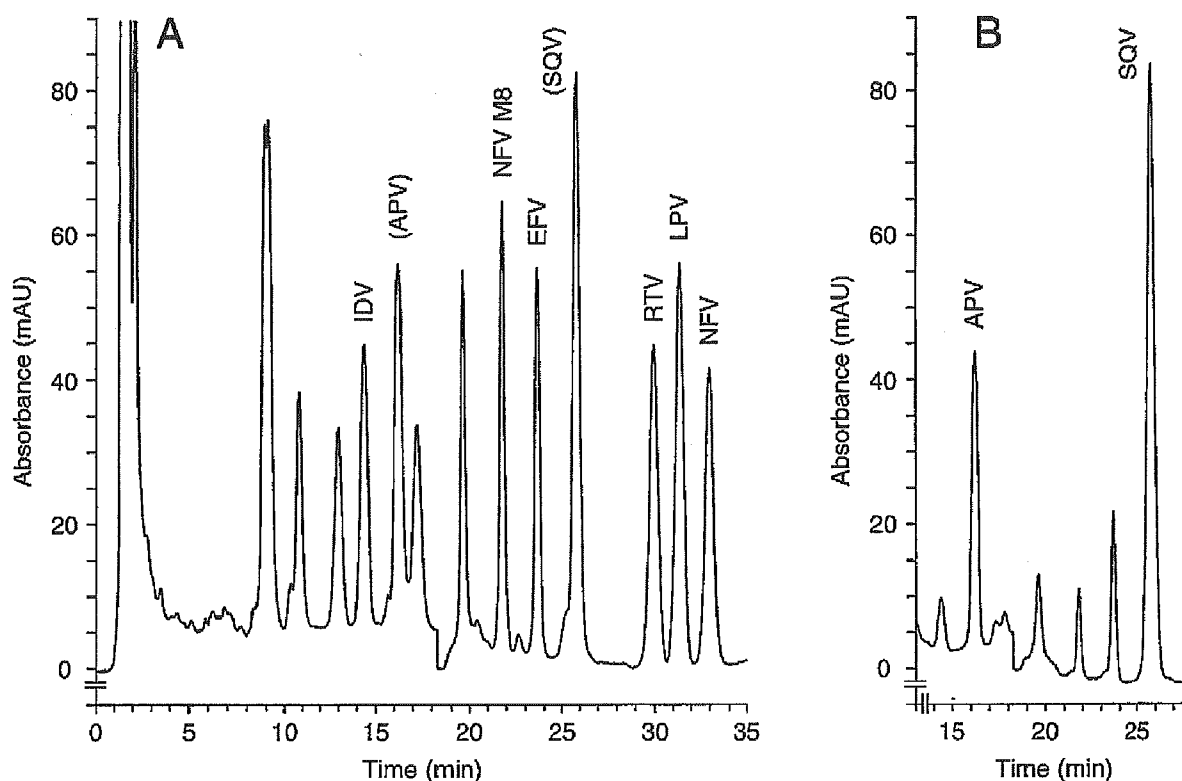

Figure 2. Chromatograms of a plasma sample spiked with 1.8 µg/mL of APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV. Absorbance was monitored at 212 nm (A) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B).

Table 1. Drugs tested for possible analytical interference

Abacavir	Erythromycin	Pentamidine
Acetaminophen	Ethambutol	Prednisolone
Aciclovir	Fluconazole	Pyrazinamide
Amikacin	Foscarnet	Rifampicin
Amoxicillin	Ganciclovir	Stavudine
Amphotericin B	Hydroxyurea	Sulfamethoxazole
Ampicillin	Isoniazid	Trimethoprim
Azithromycin	Kanamycin	Vancomycin
Cefaclor	Ketoconazole	Zalcitabine
Clarithromycin	Lamivudine	Zidovudine
Clindamycin	Metronidazole	Zidovudine glucuronide
Didanosine	Nevirapine	

which was obviously different from that of NFV M8 (21.8 min). Interference with metabolites of PIs and EFV was investigated with clinical samples, because these metabolites except NFV M8 are not available in pure form. No peaks interfering with any analyte were observed in plasma samples from patients receiving PIs and EFV (data not shown).

Over the concentration range 0.025–15 µg/mL for SQV and 0.05–15 µg/mL for the other seven analytes, the calibration curves were constructed by least-squares analysis. The correlation coefficients (r^2) of the curves were 0.995, 0.992, 0.998, 0.998, 0.997, 0.999, 0.998 and 0.999 for APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV, respectively. The results of the accuracy and precision of the method are summarized in Table 2. The accuracies for the analytes at three concentration levels ranged from −6.9 to +7.6%. The intra-assay and inter-assay precisions were <9.2 and <11.8%, respectively. The LLQs were 0.025 µg/mL for SQV and 0.05 µg/mL for the other analytes, as determined with the plasma samples spiked with decreasing concentrations of the analytes. The ULQ was arbitrarily defined as 15 µg/mL for each analyte. The accuracies and precisions at the ULQ were also <20%.

The average extraction recoveries were 84.8, 70.9, 90.6, 88.3, 73.7, 80.0, 93.1 and 95.4% for APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV, respectively. Although the reasons for the relatively lower recoveries of EFV and NFV are unclear, these had no negative effects on the assay performance as described above. The stability of the analytes in plasma samples is shown in Table 3. Under all conditions tested, the analytes proved to be stable with a recovery of >90.6% of the initial concentration.

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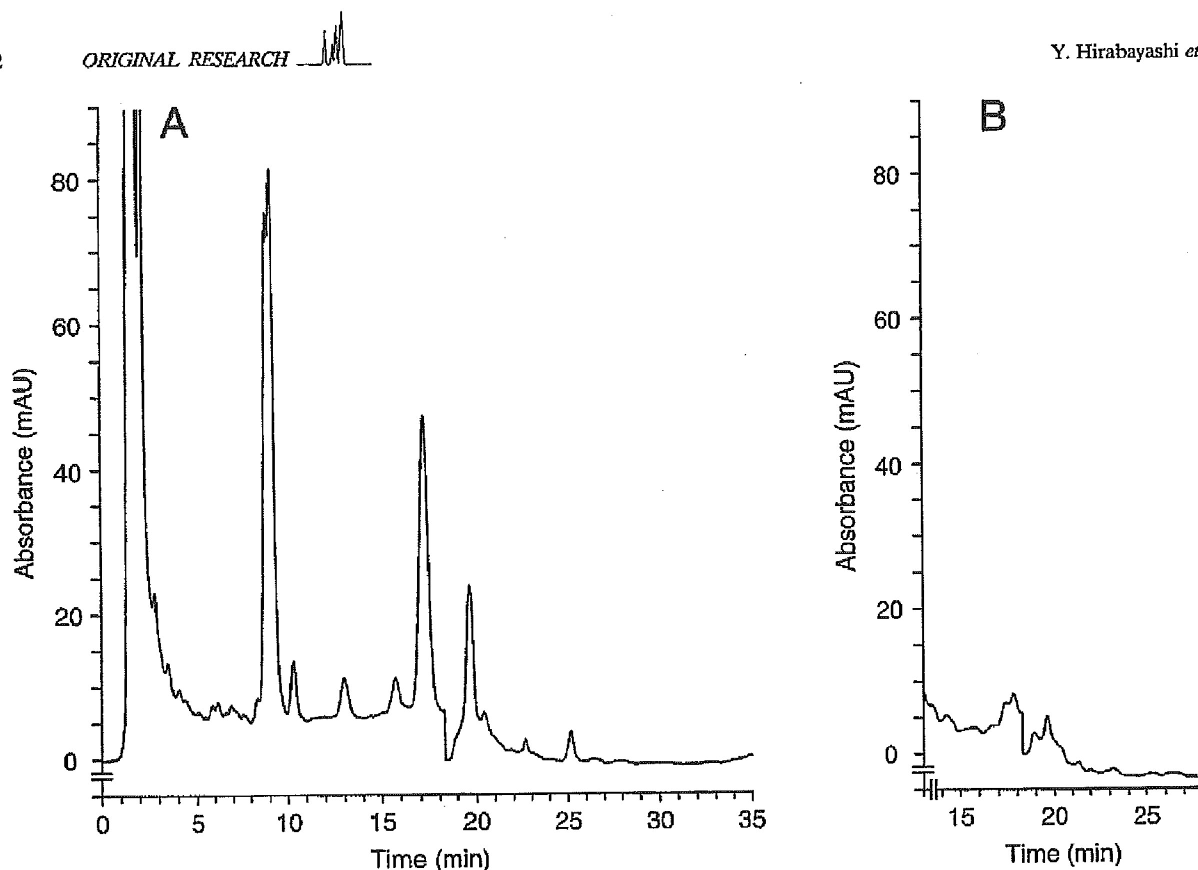

Figure 3. Chromatograms of a blank plasma sample. Absorbance was monitored at 212 nm (A) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B).

Table 2. Accuracy and precision of the analytical method

Analyte	Concentration (μg/mL)	Accuracy (%)[a] ($n = 6$)	Precision (%)[b] Intra-assay ($n = 6$)	Precision (%)[b] Inter-assay ($n = 5$)
Amprenavir	0.3	+1.3	8.9	7.8
	2.1	−6.9	4.0	5.1
	12.0	+2.0	3.9	6.7
Efavirenz	0.3	+7.6	7.5	11.8
	2.1	−0.3	4.1	8.4
	12.0	+3.5	2.2	5.3
Indinavir	0.3	−2.0	6.1	9.0
	2.1	+1.7	3.1	4.9
	12.0	+5.2	5.4	4.3
Lopinavir	0.3	+4.5	5.6	9.5
	2.1	+2.8	4.0	6.1
	12.0	−0.9	4.3	5.9
Nelfinavir	0.3	+3.1	5.0	5.3
	2.1	+1.9	5.4	4.7
	12.0	−1.6	2.1	3.2
Nelfinavir M8	0.3	+4.4	4.6	6.7
	2.1	−0.3	2.9	4.4
	12.0	−1.6	1.5	3.6
Ritonavir	0.3	−2.0	9.2	7.3
	2.1	+3.2	3.8	5.1
	12.0	−4.1	3.1	5.9
Saquinavir	0.3	+1.7	3.9	2.8
	2.1	−0.2	2.2	3.4
	12.0	+3.1	4.2	4.7

[a] Deviation from nominal concentration.
[b] Relative standard deviation within a single assay or between different assays.

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Table 3. Stability of protease inhibitors and efavirenz in plasma samples

Analyte	Concentration (μg/mL)	Recovery (%)[a]		
		60 min at 60°C ($n = 3$)	7 days at 4°C ($n = 3$)	60 days at −30°C with three freeze–thaw cycles ($n = 4$)
Amprenavir	0.3	94.7	104.8	95.6
	12.0	91.4	103.0	101.7
Efavirenz	0.3	95.8	100.7	98.8
	12.0	90.6	99.1	95.9
Indinavir	0.3	91.5	105.2	94.8
	12.0	93.2	100.8	98.6
Lopinavir	0.3	94.0	104.1	98.0
	12.0	93.3	101.3	100.5
Nelfinavir	0.3	102.4	102.5	99.8
	12.0	96.2	101.8	99.2
Nelfinavir M8	0.3	95.6	98.6	93.3
	12.0	98.1	101.8	97.6
Ritonavir	0.3	96.7	107.3	96.9
	12.0	93.2	103.0	97.2
Saquinavir	0.3	97.3	102.9	97.8
	12.0	92.8	102.6	100.4

[a] Compared with freshly prepared samples.

Table 4. Plasma concentrations of protease inhibitors and efavirenz in 10 patients

Patient no.	Drug	Dose (mg)[a]	Time after dosing (h)	Concentration (μg/mL)
1	Amprenavir	1200, b.i.d.	10.0	0.22
	Efavirenz	600, q.d.	10.0	0.53
2	Amprenavir	1200, b.i.d.	2.0	6.47
	Ritonavir	200, b.i.d.	2.0	0.38
	Efavirenz	600, q.d.	12.0	1.47
3	Indinavir	800, t.i.d.	6.5	0.19
4	Indinavir	400, b.i.d.	2.0	3.53
	Ritonavir	400, b.i.d.	2.0	2.55
5	Lopinavir	400, b.i.d.	3.0	5.94
	Ritonavir	100, b.i.d.	3.0	0.42
6	Lopinavir	400, b.i.d.	12.0	3.06
	Ritonavir	100, b.i.d.	12.0	0.09
7	Nelfinavir	1250, b.i.d.	4.5	1.01
			(nelfinavir M8 0.96)	
8	Nelfinavir	1250, b.i.d.	11.0	0.78
			(nelfinavir M8 0.23)	
9	Saquinavir	400, b.i.d.	3.5	1.49
	Ritonavir	400, b.i.d.	3.5	6.03
10	Saquinavir	400, b.i.d.	11.5	0.56
	Ritonavir	400, b.i.d.	11.5	1.30

[a] b.i.d., twice a day; q.d., once a day; t.i.d., three times a day.

The present method was applied to the determination of drug concentrations in clinical samples (Table 4 and Fig. 4). Plasma samples were randomly collected from 10 patients receiving PIs and EFV. All the samples could be analyzed with no technical difficulties. The observed concentrations were within the concentration ranges reported in literature (Khoo *et al.*, 2001; Acosta *et al.*, 2002; Back *et al.*, 2002; Van Heeswijk, 2002).

DISCUSSION

In this report, we describe the development, validation and clinical application of a novel chromatographic method for the simultaneous determination of the six widely used PIs and the most frequently used NNRTI, EFV, in plasma. To date, several methods have been reported for the simultaneous determination of different PIs or PIs plus NNRTIs (Leibenguth *et al.*, 2001;

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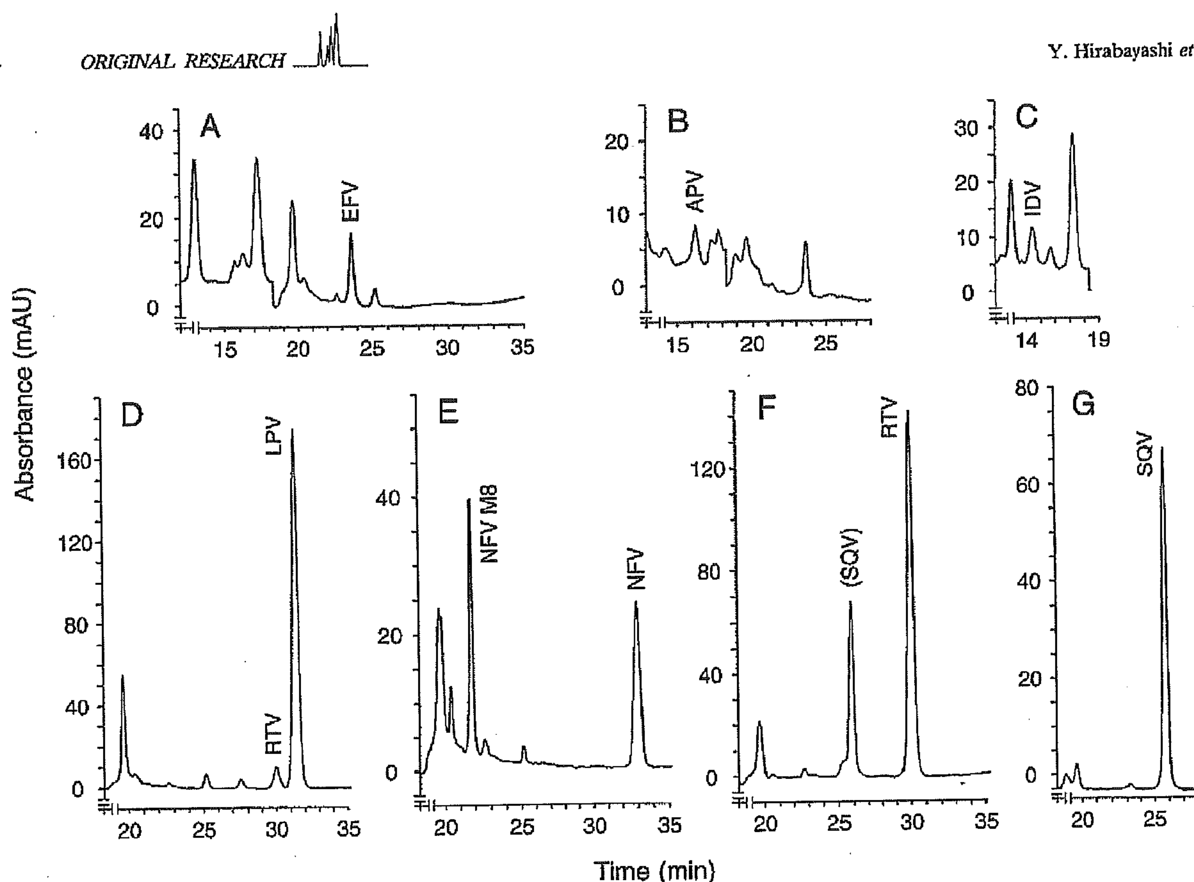

Figure 4. Chromatograms of plasma samples from patient 1 (A and B), patient 3 (C), patient 5 (D), patient 7 (E) and patient 9 (F and G). Absorbance was monitored at 212 nm (A, C, D, E and F) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B and G). For details of the patients, see Table 4.

Poirier *et al.*, 2002; Titier *et al.*, 2002; Tribut *et al.*, 2002; Volosov *et al.*, 2002; Crommentuyn *et al.*, 2003; Frerichs *et al.*, 2003; Rentsch, 2003; Turner *et al.*, 2003; Rezk *et al.*, 2004). However, most of these methods have limitations in clinical application, including insufficient quantitation sensitivity, laborious sample pretreatment with solid-phase extraction, or use of expensive mass spectrometry. A simple, economical and reliable method that is performable with standard hospital laboratory equipment is desirable for routine TDM. The present method proved sufficiently sensitive to be used for TDM, because the LLQ values of the method for individual drugs were lower than the trough concentrations observed with treated patients and the target trough concentrations estimated from clinical and *in vitro* data (Khoo *et al.*, 2001; Acosta *et al.*, 2002; Back *et al.*, 2002; Van Heeswijk, 2002). The method was also accurate and precise over a wide range of drug concentrations as described. Chromatography, which was relatively time-consuming, could be fully automated with no need for technical supervision, since samples were stable for 24 h in the auto-sampler (data not shown). We utilized an ultraviolet detector, commonly used in hospital laboratories, but not expensive and sophisticated mass spectrometry. For the sample pretreatment, we chose liquid–liquid extraction, which is economical compared with solid-phase extraction. To simplify the experimental procedure, an internal standard was not used; nevertheless the method provided satisfactory validation results. Thus, this method would be suitable for routine TDM in conventional hospital laboratory settings.

Moreover, from a clinical point of view, this method is noteworthy for two reasons. First, this method covers most of key drugs currently used other than NRTIs. Since the standard treatment of HIV infection, HAART, consists of various combinations of anti-HIV drugs (Richman, 2001; British HIV Association Writing Committee, 2003; The Panel on Clinical Practices for Treatment of HIV Infection, 2004; Yeni *et al.*, 2004), it would be convenient to use TDM to measure plasma concentrations of several drugs simultaneously with a single method rather than to use each different method for each drug. Although NRTI concentrations cannot be determined with this method, NRTIs are pro-drugs that are converted to their active triphosphate forms within cells, and therefore TDM with plasma is considered less beneficial compared with PIs and NNRTIs,

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which directly exert their antiviral effects (Back *et al.*, 2002). Secondly, this method allows the determination of the M8 metabolite of NFV as well as the parent NFV. NFV M8, which is produced by CYP 2C19 in the liver, is equipotent to the unchanged parent NFV against HIV *in vitro* (Zhang *et al.*, 2001), although almost all the metabolites of PIs and EFV have no obvious antiviral activity. The pharmacokinetics of NFV M8 would be also markedly affected by genetic background, drug interactions and hepatic dysfunction, leading to a wide inter-patient variability (Khaliq *et al.*, 2000). These findings suggest that measurement of the parent NFV plus its M8 metabolite in plasma would be preferable to that of the parent alone for TDM in NFV treatment.

TDM with this method is expected to contribute to the optimization of HIV treatment for individual patients through modification of dosage and assessment of adherence to treatment. This method would be also useful for studying the relationships between drug concentrations and efficacy or toxicity and for analyzing pharmacokinetics and drug interactions in heavily co-medicated patients. Such concentration-oriented approaches and studies are in progress in our hospital.

CONCLUSION

A simple, economical and reliable chromatographic method has been developed for the simultaneous determination of the six PIs, NFV M8 and EFV in plasma. This method would be useful for routine TDM and pharmacokinetic studies in patients receiving PIs and EFV.

Acknowledgments

The authors thank Hiroyuki Fukuda of Applied Biosystems Japan Ltd for helpful suggestions. The authors also thank Abbott Laboratories, Japan Tobacco Inc., Kissei Pharmaceutical Co., Merck & Co. and Roche Products for providing the reference compounds. This study was supported in part by Grants-in-Aid for AIDS research from the Ministry of Health, Labour and Welfare of Japan (H15-15100101), by the Japanese Foundation for AIDS Prevention, and by the Organization of Pharmaceutical Safety and Research (01-4).

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Virology 344 (2006) 354 – 362

VIROLOGY

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Mutations other than 103N in human immunodeficiency virus type 1 reverse transcriptase (RT) emerge from K103R polymorphism under non-nucleoside RT inhibitor pressure

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Received 19 June 2005; returned to author for revision 29 July 2005; accepted 7 September 2005
Available online 10 October 2005

Abstract

K103N mutation in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) confers high-level resistance against non-nucleoside RT inhibitors (NNRTIs) and it easily occurs partly because it arises by a single nucleotide substitution from wild-type K103. There are polymorphisms at codon 103 of HIV-1 RT. We found K103R polymorphic mutation in 3.3% of treatment-naive HIV-1-infected patients. R103N does not seem to occur as easily as K103N because R103N requires two nucleotide substitutions. To induce NNRTI resistance-associated mutations, HIV-1_{K103R} was propagated in the presence of increasing concentrations of efavirez (EFV) or nevirapine (NVP). V179D emerged in all three EFV cultures and in two of four NVP cultures. R103G emerged by a single nucleotide substitution in one of three EFV cultures. R103N did not emerge in any of 7 NNRTI cultures. Analysis of recombinant HIV-1s showed that HIV-$1_{K103R/V179D}$ was significantly resistant and HIV-1_{K103G} was moderately resistant against EFV and NVP.
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Keywords: K103R; Polymorphic; Mutation; Non-nucleoside reverse transcriptase inhibitor; Resistance; HIV-1

Introduction

The emergence of human immunodeficiency virus type 1 (HIV-1) variants resistant to antiretroviral agents is one of the most common causes for therapeutic failure in infected individuals. Fortunately, the availability of drug resistance testing has substantially improved the ability of clinicians to deal knowledgeably with drug-resistant HIV-1 strains (Vandamme et al., 2004). Recent studies, however, have revealed that certain polymorphic amino acid residues of HIV-1 contribute to drug resistance (Gatanaga et al., 2002; Harrigan et al., 2005; Tanaka et al., 1997). Even if polymorphic mutations themselves do not alter drug susceptibility, their combination with other unreported mutations might confer significant drug resistance. Such combinations are probably not included in the current reference tables of drug resistance-associated mutations and the effect of preexisting polymorphic mutations on the development of drug resistance is not well taken into consideration (Johnson et al., 2005).

Non-nucleoside reverse transcriptase inhibitor (NNRTI)-containing combination of antiretroviral agents are recommended as the first-line regimens for HIV-1-infected individuals (Yeni et al., 2004). One of the most critical problems, however, is the easy emergence of NNRTI resistance-associated mutations, among which the K103N mutation in reverse transcriptase (RT) is the most common and important (Bacheler, 1999; De Clercq, 1998; Harrigan et al., 2005; Nunberg et al., 1991). However, there are polymorphic mutations at codon 103 of HIV-1 RT (Harrigan et al., 2005), and their effect on emergence of drug resistance is not well studied. In this study, we analyzed the polymorphic frequencies at codon 103 of HIV-1 RT in treatment-naive patients and determined the effect of such polymorphism on the development of NNRTI resistance.

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doi:10.1016/j.virol.2005.09.005

Results

Frequency of K103R in treatment-naïve patients

We analyzed HIV-1 sequences in plasma samples of 211 antiretroviral treatment-naive infected individuals who visited our clinic from January, 2000 to December, 2003, and found wild-type K103 in 204 patients (96.7%; nucleotide triplet: AAA in 202 samples, AAG in 2 samples) and K103R in six (2.8%; nucleotide triplet: all AGA) and a mixture of these in one (0.5%; nucleotide triplet: AAA and AGA) (Table 1). Taken together, K103R was detected in 3.3% of our treatment-naive patients by direct sequencing.

Induction of EFV-resistant HIV-1 variants

The K103N mutation can arise by a single nucleotide substitution (AAA to AAC or AAT, AAG to AAC or AAT) from wild-type K103, while the amino acid substitution from arginine (R) to asparagine (N) at codon 103, R103N, necessitates at least two nucleotide substitutions (AGA to AAC or AAT). Therefore, R103N does not seem to occur as easily as K103N. In order to find the types of mutations that can be induced by NNRTI from K103R, the infectious HIV-1 clones harboring wild-type K103 (HIV-1_{WT}) and K103R (HIV-1_{K103R}) in RT were propagated in MT-2 cells in the presence of increasing concentrations of EFV, respectively. The culture supernatant was harvested on day 7 of culture and used to infect fresh MT-2 cells for the next round of culture. When the virus began to propagate in the presence of the drug, the drug concentration was increased. HIV-1_{WT} harboring nucleotide triplet AAA at codon 103 of RT, was propagated in two independent cultures (Figs. 1A and B). In one culture, I178M mutation first emerged at 11 weeks of passages and K103N was added to it at 22 weeks (Fig. 1A). In the other culture, Y181C first emerged at 6 weeks, and K101E and V179D were combined to it at 16 weeks (Fig. 1B). Furthermore, V106I and V108I were added to them at 20 weeks, but V106I could not be detected by direct sequencing at 25 weeks (Fig. 1B). In order to determine the effect of synonymous nucleotide substitution on the development of EFV resistance, HIV-1_{WT} harboring nucleotide triplet AAG at codon 103 of RT, was also propagated in the same way (Fig. 1C). K103N emerged at 12 weeks, and L100I was combined to it at 25 weeks. Considered together, these results indicated it was easy for K103N to emerge as it arose in two of three cultures of HIV-1_{WT} under the selective pressure of EFV.

HIV-1_{K103R} was propagated in three independent cultures (Figs. 1D–F). In one culture, G190A emerged at 11 passages, and V179D was added at 25 weeks (Fig. 1D). In another culture, R103G, which has not been reported previously, arose by a single nucleotide mutation (nucleotide triplet: AGA to GGA) at 8 weeks, and V106I emerged at 11 weeks (Fig. 1E). At 22 weeks, V106I could not be detected by direct sequencing and V108I, V179D, and G190A emerged. Finally, R103G and V108I could not be detected and V106I re-emerged at 25 weeks (Fig. 1E). In the last culture, V179D emerged at 14 weeks and no other mutations were added to it until the EFV concentration reached 1000 nM at 25 weeks, suggesting that the combination of K103R and V179D conferred high-level resistance to EFV (Fig. 1F). In conclusion, R103N mutation was not detected in any of the three culture of HIV-1_{K103R}, although it often arose in cultures of HIV-1_{WT}. Instead of R103N, R103G occurred by a single nucleotide mutation in one culture. V179D emerged in all of three cultures of HIV-1_{K103R} and the combination of K103R and V179D seemed to confer high-level resistance to EFV.

Clonal determination of amino acid mutations in EFV-resistant HIV-1 variants

In one culture of HIV-1_{K103R} described above (Fig. 1E), 103G mutation, which has not been reported previously, and V106I mutation, which is polymorphic (Rhee et al., 2003), were detected at early passages and the mutation pattern was complicated with the appearance and disappearance of various mutations at late passages. Therefore, we decided to analyze the mutations by cloning at several passages (Table 2). At 14 weeks when EFV concentration had not yet increased, R103G and V106I emerged in independent clones. However, the V106I clone without any other acquired mutation was not detected and every analyzed clone had R103G at 18 weeks when EFV concentration was increased to 30 nM. These results indicate that V106I itself conferred little resistance to EFV to HIV-1_{K103R} and that R103G conferred moderate resistance. At 20 weeks when EFV concentration was further increased to 100 nM, 19 of 20 clones had R103G, and one clone had V106I and V179D. The latter clone still existed at 22 weeks (EFV: 500 nM) at a frequency of 2 of 21, which suggests that the addition of V106I and V179D to HIV-1_{K103R} conferred high-level resistance to EFV. At 25 weeks (EFV: 1000 nM), the clone with V106I, V179D, and G190A predominated other clones. Considered together, the results indicate that V106I clone without any other acquired mutation existed only at the initial EFV concentration (3 nM), and once EFV concentration was increased, every analyzed clone had R103G or the combination of K103R, V106I, and V179D, suggesting that V106I itself conferred little EFV resistance to HIV-1_{K103R} and that R103G and the combination K103R, V106I, and V179D conferred significant resistance to EFV.

Table 1
Frequency of mutations at codon 103 of HIV-1 RT in treatment-naive patients

Amino acid (nucleotides)	Number of patients
K (AAA)	202
K (AAG)	2
R (AGA)	6
K (AAA)/R (AGA)[a]	1
Total	211

Deduced amino acids and nucleotide triplets at codon 103 of HIV-1 RT are shown.

[a] Mixture of K (AAA) and R (AGA).

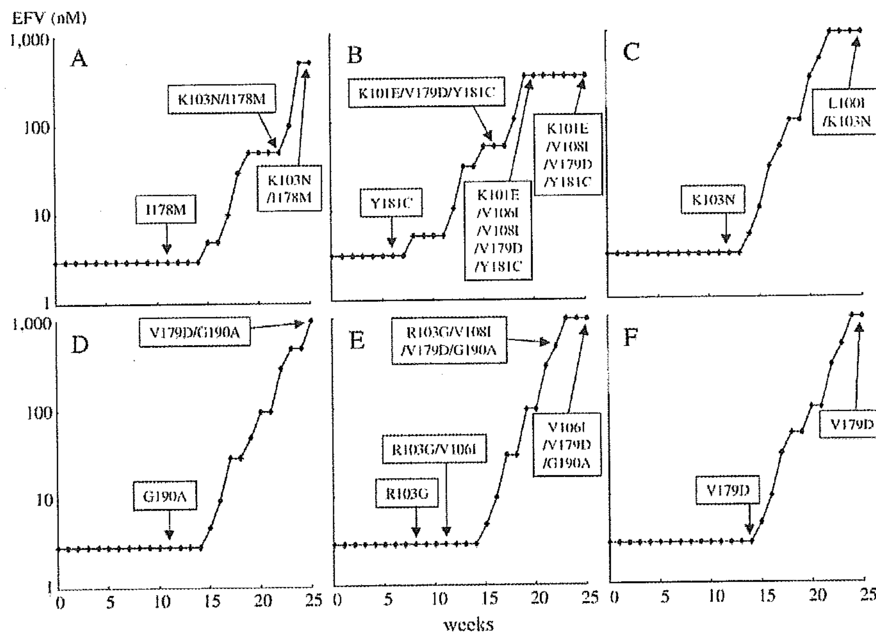

Fig. 1. Induced amino acid substitutions under the selective pressure of EFV. HIV-1_{WT} harboring nucleotide triplet AAA (panels A and B), and AAG (panel C) at codon 103 of RT, and HIV-1_{K103R} (panels D–F) were propagated in MT-2 cells in the presence of increasing concentrations of EFV. Induced amino acid substitutions were analyzed at several passages by sequencing proviral HIV-1 RT in MT-2 cells.

Induction of NVP-resistant HIV-1 variants

Under the selective pressure of EFV, R103N did not emerge but the unreported R103G appeared instead, and V179D developed in every culture of HIV-1_{K103R}. To obtain further insight on the development of NNRTI resistance from HIV-1_{K103R}, we passaged HIV-1_{K103R} in the presence of increasing concentrations of NVP. HIV-1_{K103R} was propagated in four independent cultures (Figs. 2A–D). In one culture, V179D emerged first at 10 weeks and V106A, which is well known to confer significant resistance to NVP (Johnson et al., 2005), was added to it at 19 weeks (Fig. 2A). In the other three cultures (Figs. 2B–D), V106A emerged first at 8–10 weeks, and V179D was combined to it at 16 weeks in one culture (Fig. 2C). In summary, R103N did not emerge in any of the four cultures of HIV-1_{K103R} as expected, and V106A appeared in every culture. V179D, which was observed in all three cultures with EFV, emerged in two of four cultures with NVP, suggesting that the combination of K103R and V179D conferred significant resistance not only to EFV but also to NVP and increased the viral replication cooperated with V106A in the presence of NVP.

Table 2
Clonal sequence analysis of RT gene of HIV-1 passaged in the presence of EFV (Fig. 1E)

Weeks (EFV)	Amino acid						Clones/ total
	103 (R)	106 (V)	108 (V)	135 (I)	179 (V)	190 (G)	
14 (3 nM)	–	–	–	–	–	–	7
	G	–	–	–	–	–	7
	–	I	–	–	–	–	3/17
18 (30 nM)	G	–	–	–	–	–	21
	G	I	–	–	–	–	3/24
20 (100 nM)	G	I	–	–	–	–	10
	G	–	–	–	–	–	9
	–	I	–	–	D	–	1/20
22 (500 nM)	G	–	I	–	–	A	14
	G	–	–	–	D	–	2
	–	I	–	–	D	–	2
	G	–	–	–	–	A	1
	G	–	I	–	D	A	1
	G	–	I	V	D	–	1/21
23 (1000 nM)	–	I	–	–	D	A	7
	G	–	–	V	D	–	6
	G	–	I	–	–	A	5
	G	–	I	–	D	A	1
	G	–	–	V	D	A	1/20
25 (1000 nM)	–	I	–	–	D	A	20/20

Data are deduced amino acids at the positions where mutations were detected. Identity with original amino acids shown at the top is indicated by the dash sign.

NNRTI resistance conferred by K103R/V179D and K103G

In order to analyze the effect of each mutation and their combinations on drug resistance, we constructed a panel of recombinant HIV-1 clones and determined their IC_{50} for four NRTIs, including zidovudine (AZT), lamivudine (3TC), stavudine (d4T), and tenofovir disoproxil fumarate (TDF), and two NNRTIs, including EFV and NVP. Drug resistance assay was performed in triplicate using MAGIC-5 cells (Table 3) (Hachiya et al., 2001, 2003, 2004). As reported previously, K103R and V106I alone did not confer significant drug resistance (Harrigan et al., 2005; Rhee et al., 2003). Furthermore, their combination, K103R/V106I, did not exhibit significant drug resistance, although it was observed

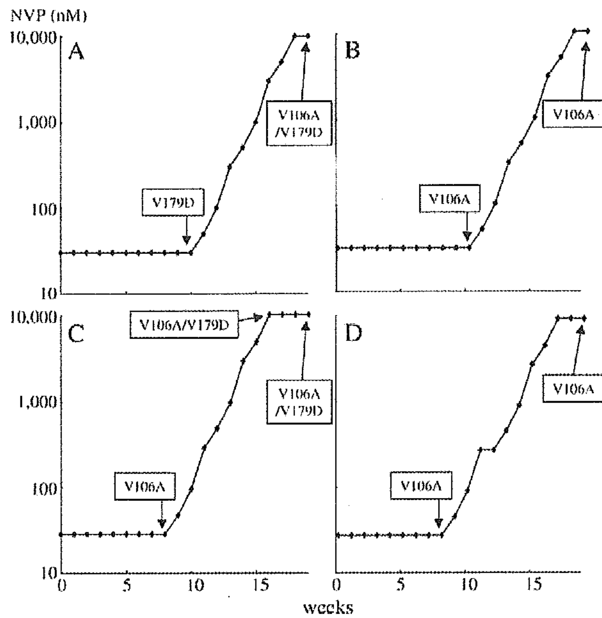

Fig. 2. Induced amino acid substitutions under the selective pressure of NVP. HIV-1_{K103R} was propagated in four cultures (panels A–D) of MT-2 cells in the presence of increasing concentrations of NVP. Induced amino acid substitutions were analyzed at several passages by sequencing proviral HIV-1 RT in MT-2 cells.

during passages in one culture under EFV selective pressure (Fig. 1E). The previously unreported mutation K103G, which emerged in one culture of HIV-1_{K103R} under EFV pressure, conferred 5.60- and 4.71-fold resistance to EFV and NVP, respectively. The combination of K103R and V179D, which was observed in all three cultures of HIV-1_{K103R} under EFV pressure and in two of four cultures under NVP pressure, conferred 10.4- and 9.41-fold resistance to EFV and NVP, respectively, though V179D alone had little effect on EFV resistance (1.60-fold) but moderate resistance only to NVP (5.10-fold). Therefore, as expected from the data of emergence patterns of induced mutations under NNRTI selective pressure, K103G conferred moderate resistance to EFV and NVP, and the combination of K103R and V179D conferred high-level resistance to these NNRTIs.

Decreased replication kinetics of HIV-$1_{K103R/V179D}$

Finally, we analyzed the effect of single mutations and their combinations on HIV-1 replication kinetics in MT-2 cells in the absence and presence of NNRTI (Fig. 3 and Table 4). Each replication assay was performed in triplicate and repeated three times. In the absence of NNRTI, K103R and V106I did not significantly alter HIV-1 replication. Their combination (K103R/V106I), however, significantly reduced viral replication (p24 at day 8 of K103R/V106I vs. wild-type: $P < 0.05$, Fig. 3A and Table 4). K103G also resulted in reduced replication (K103G vs. wild-type: $P < 0.05$). V179D tended to reduce HIV-1 replication but the effect was not statistically significant in our assay. The combination of K103R and V179D severely compromised HIV-1 replication (K103R/V179D vs. wild-type: $P < 0.01$, Fig. 3A and Table 4). As reported previously, K103N did not significantly decrease HIV-1 replication (Gianotti et al., 2004; Schmit et al., 1996).

In the presence of 10 nM EFV, HIV-1 clones harboring wild-type K103 (HIV-1_{WT}), K103R (HIV-1_{K103R}), V106I (HIV-1_{V106I}), and K103R/V106I (HIV-$1_{K103R/V106I}$), failed to propagate and HIV-1 harboring V179D (HIV-1_{V179D}) exhibited reduced replication compared with that in the absence of NNRTI ($P < 0.05$, Fig. 3B and Table 4). HIV-1 clones harboring K103N (HIV-1_{K103N}), K103G (HIV-1_{K103G}), and the combination of K103R and V179D (HIV-$1_{K103R/V179D}$) showed efficient replication though their replication was slightly reduced compared with those in the absence of NNRTI. In the presence of 100 nM NVP, HIV-1_{K103N}, HIV-1_{K103G}, HIV-1_{V179D}, and HIV-$1_{K103R/V179D}$ exhibited efficient replications (Fig. 3C). The replication of the other HIV-1 clones was severely compromised. These data of the replication kinetics were well compatible with the IC_{50} data (Table 3).

Competitive HIV-1 replication assay using H9 cells

In order to confirm some of the results shown above, competitive HIV-1 replication assay was performed using H9 cells (Hachiya et al., 2004; Kosalaraksa et al., 1999) (Fig. 4). Two infectious HIV-1 clones to be compared for their fitness were mixed and used to infect H9 cells, and their population changes through weekly passages were determined by the

Table 3
Drug susceptibilities of recombinant HIV-1s

HIV-1	Mean IC_{50} (nM) ± SD (fold resistance)					
	AZT	3TC	d4T	TDF	EFV	NVP
Wild-type	30.0 ± 3.5	775 ± 14	1510 ± 200	27.0 ± 2.8	2.50 ± 0.25	25.5 ± 3.6
K103R	17.0 ± 1.2 (0.567)	340 ± 32 (0.439)	1500 ± 120 (0.993)	23.0 ± 1.9 (0.852)	2.40 ± 0.34 (0.960)	19.0 ± 2.2 (0.745)
K103N	14.0 ± 1.3 (0.467)	380 ± 27 (0.490)	1400 ± 130 (0.927)	29.0 ± 2.2 (1.07)	31.0 ± 2.3 (12.4)	1100 ± 130 (43.1)
V106I	19.0 ± 2.4 (0.633)	860 ± 33 (1.11)	1090 ± 70 (0.722)	31.0 ± 2.0 (1.15)	3.00 ± 0.33 (1.20)	20.0 ± 1.2 (0.784)
K103G	24.0 ± 1.0 (0.800)	1160 ± 30 (1.50)	660 ± 100 (0.437)	24.0 ± 2.0 (0.889)	14.0 ± 1.3 (5.60)	120 ± 10 (4.71)
K103R/V106I	29.0 ± 1.3 (0.967)	1420 ± 80 (1.83)	1200 ± 330 (0.795)	31.0 ± 1.3 (1.15)	3.00 ± 0.33 (1.20)	30.0 ± 11 (1.18)
V179D	16.0 ± 1.5 (0.533)	830 ± 23 (1.07)	3600 ± 230 (2.38)	36.0 ± 3.0 (1.33)	4.00 ± 0.17 (1.60)	130 ± 20 (5.10)
K103R/V179D	34.0 ± 2.0 (1.13)	1980 ± 12 (2.55)	1430 ± 140 (0.947)	29.0 ± 2.7 (1.07)	26.0 ± 2.0 (10.4)	240 ± 12 (9.41)

Drug resistance assay was performed in triplicate using MAGIC-5 cells (Hachiya et al., 2001, 2003, 2004). Fold resistance was calculated by dividing IC_{50} of recombinant HIV-1 by that of wild-type HIV-1.

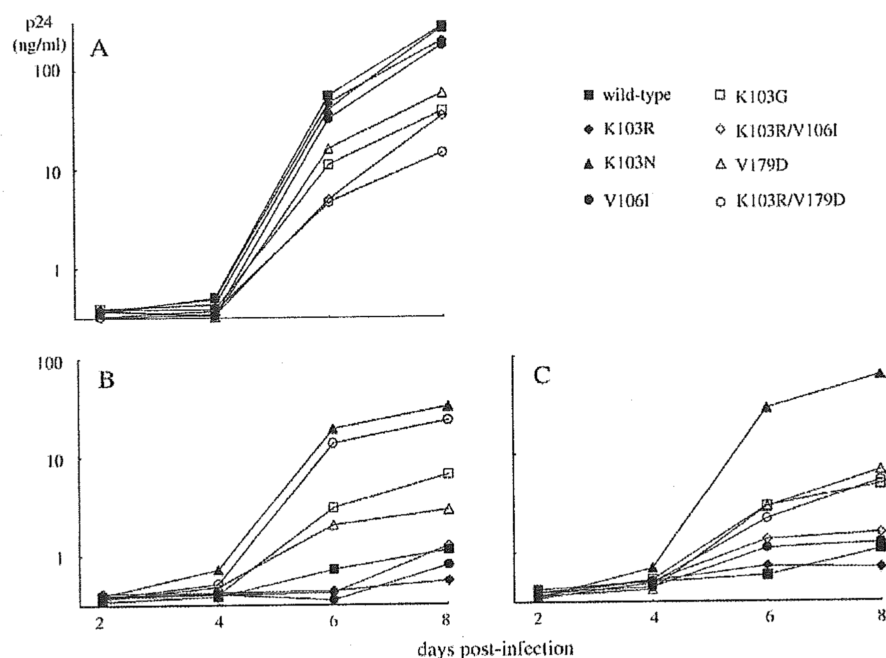

Fig. 3. Replication kinetics of recombinant HIV-1 clones in the absence and presence of NNRTIs. Recombinant HIV-1 clones were propagated in MT-2 cells in the absence (panel A) and presence of 10 nM EFV (panel B) or 100 nM NVP (panel C). The concentration of p24 in the culture medium was measured every other day. The assay was performed in triplicate and repeated three times and the data represent the logarithmic mean values of p24 concentrations.

relative peak height on sequencing electrogram. In the absence of NNRTI (Fig. 4A), and in the presence of EFV (3 nM) (Fig. 4B) and NVP (30 nM) (Fig. 4C), HIV-1_{WT} and HIV-1_{K103R} showed comparable replication efficiency, though the replication of HIV-1_{K103R} may be a little smaller than that of HIV-1_{WT} in the absence of NNRTI and in the presence of NVP. HIV-1_{K103N} was slowly outgrown by HIV-1_{WT} in the absence of NNRTI and it was still detectable at 3 weeks of competitive culture (wild-type:K103N = 91%:9%) (Fig. 4D). In the presence of EFV (10 nM) (Fig. 4E) and NVP (100 nM) (Fig. 4F), however, HIV-1_{K103N} readily outgrew HIV-1_{WT}. HIV-$1_{K103R/V179D}$ was predominated by HIV-1_{WT} in the absence of NNRTI (Fig. 4G), though it readily outgrew HIV-1_{WT} in the presence of EFV (10 nM) (Fig. 4H) and NVP (100 nM) (Fig. 4I). These data of competitive replication assay were well compatible with replication kinetic data (Fig. 3) and confirmed that the combination of K103R and V179D reduced viral replication in the absence of NNRTI though K103R alone had little impact on viral replication ability.

Table 4
Replication ability of recombinant HIV-1s

HIV-1	Logarithmic mean ± SD of p24 (ng/ml) at day 8		
	No drug	10 nM EFV	100 nM NVP
Wild-type	2.437 ± 0.309	0.021 ± 0.134	−0.003 ± 0.257
K103R	2.289 ± 0.343	−0.284 ± 0.102	−0.188 ± 0.266
K103N	2.422 ± 0.273	1.477 ± 0.287	1.759 ± 0.257
V106I	2.243 ± 0.777	−0.128 ± 0.137	0.063 ± 0.105
K103G	1.579 ± 0.285	0.789 ± 0.571	0.658 ± 0.399
K103R/V106I	1.534 ± 0.245	0.064 ± 0.096	0.164 ± 0.096
V179D	1.764 ± 0.450	0.431 ± 0.347	0.805 ± 0.288
K103R/V179D	1.159 ± 0.330	1.339 ± 0.208	0.697 ± 0.315

Recombinant HIV-1 clones were propagated in MT-2 cells in the absence and presence of 10 nM EFV or 100 nM NVP (Fig. 3). The assay was performed in triplicate and repeated three times. The logarithmic mean values and SD of p24 concentrations in the culture media at day 8 were shown.

Discussion

K103R mutation in RT was detected in 3.3% of our treatment-naive patients and was not associated with NNRTI resistance in our recombinant HIV-1 experiments. It was also reported that K103R was most often observed in individuals not receiving NNRTI among patients with known treatment history (Harrigan et al., 2005). Taken together, K103R can be considered a naturally occurring polymorphism in HIV-1 RT and is not associated with NNRTI exposure or resistance. K103N is the most commonly observed NNRTI resistance-associated mutation, which is considered to arise by a single nucleotide mutation from wild-type K103. However, R103N, the same amino acid substitution from K103R, which necessitates two nucleotide substitutions, did not occur in our in vitro induction of NNRTI resistance. Instead, a previously unreported mutation, R103G, which consisted of a single nucleotide substitution, was observed and shown to confer moderate resistance to EFV and NVP. It is worth noting that 103S, a rare mutation, was reported to follow K103R in three cases in the British Columbia database and was shown to confer high-level resistance to NNRTIs by analyzing recombinant HIV-1s (Harrigan et al., 2005). R103S mutation can also arise by a single nucleotide substitution from K103R, though

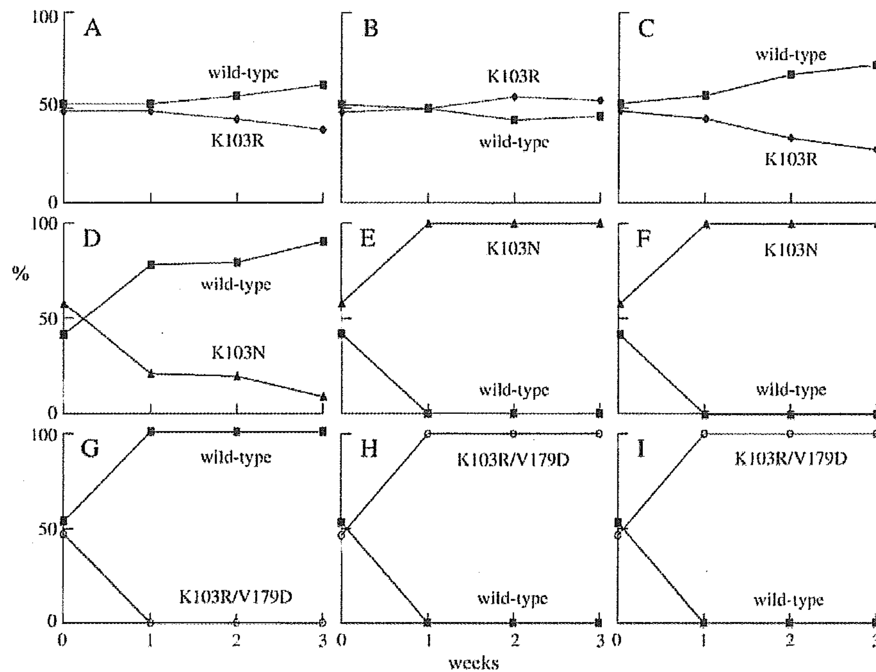

Fig. 4. Competitive HIV-1 replication assay in H9 cells. Two infectious HIV-1 clones to be compared for their fitness (HIV-1_{WT} vs. HIV-1_{K103R} in panels A–C, HIV-1_{WT} vs. HIV-1_{K103N} in panels D–F, and HIV-1_{WT} vs. HIV-$1_{K103R/V179D}$ in panels G–I) were mixed and used to infect H9 cells in the absence (panels A, D, and G) and presence of EFV (3 nM in panel B, 10 nM in panels E and H) or NVP (30 nM in panel C, 100 nM in panels F and I). The cell-free supernatant was transferred to fresh H9 cells every 7 days. High-molecular-weight DNAs extracted from infected cells on day 1 of the culture (0 week) and at the end of each passage (1, 2, and 3 weeks) were subjected to nucleotide sequencing, and proportion of Lys and Arg at position 103 (panels A–C and G–I), those of Lys and Asn at position 103 (panels D–F), and those of Val and Asp at position 179 (panels G–I) were determined.

K103S necessitates at least two nucleotide substitutions. Virological analysis of such polymorphism-specific mutations seems important, considering that the prevalence of 103S increased over fourfold in both Virco and British Columbia databases from 1998 to 2002 (Harrigan et al., 2005).

V179D is not included in the current table of drug resistance mutations by the International AIDS Society-USA Drug Resistance Mutations Group (Johnson et al., 2005), though it was reported to confer a small resistance to NNRTIs (Byrnes et al., 1993; Kleim et al., 1996; Winslow et al., 1996; Young et al., 1995). In the present study, V179D was often induced by NNRTIs in HIV-1 harboring K103R. In Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu/index.html), there are 57 registered clinical isolates harboring K103R and V179D mutations, and most of them are derived from the patients with a history of NNRTI treatment, which indicates that our in vitro observation actually often occurs in the patients. The combination of K103R and V179D conferred high-level resistance to EFV and NVP in our assay, suggesting that certain polymorphic mutation can alter the frequency of emergence of resistance-associated mutations and that the combination of a certain polymorphic mutation and a minor resistance-associated mutation can confer high-level resistance even if the polymorphism itself is not associated with drug resistance. The combination of K103R and V179D complicated viral replication though K103R alone did not significantly alter replication kinetics (Figs. 3A and 4A, G). These results suggest that HIV-$1_{K103R/V179D}$ might disappear rapidly and be replaced by HIV-1_{K103R} or HIV-1_{WT} after the discontinuation of NNRTI treatment, and that routine genotypic assay might not detect this combination in patients during NNRTI-free periods even if V179D emerged in patients during NNRTI-based therapy. On the other hand, K103N did not severely impair HIV-1 replication (Figs. 3A and 4D), in agreement with the recent studies reporting that HIV-1_{K103N} replicates comparably with HIV-1_{WT} and that K103N mutation remains stably detectable for a long time after the cessation of the NNRTI administration (Collins et al., 2004; Gianotti et al., 2004; Schmit et al., 1996).

Certain polymorphic amino acid residues in HIV-1 strains are associated with HIV-1 drug resistance (Gatanaga et al., 2002; Harrigan et al., 2005; Tanaka et al., 1997). It is also known that certain drug resistance-conferring amino acid substitutions found in one subtype HIV-1 isolated from patients under therapy may be detected in HIV-1 of other subtypes from untreated individuals (Cornelissen et al., 1997; Quinones-Mateu et al., 1998). Moreover, a recent study revealed that HIV-2 strains harbor specific patterns of natural polymorphism and resistance (Colson et al., 2004). More attention should be given to resistance-associated mutations that are specifically derived from certain polymorphisms and certain subtypes. In vitro induction of drug resistance to HIV strains harboring such polymorphisms and subtypes is a reasonable strategy for analyzing such mutations.

Materials and methods

HIV-1 sequences in treatment-naive patients

We analyzed HIV-1 sequences in plasma samples of 211 antiretroviral treatment-naive infected individuals who visited the outpatient clinic of the AIDS Clinical Center, International Medical Center of Japan from January, 2000 to December, 2003. The Institutional Review Board approved this study (IMCJ-H13-80) and a written informed consent was obtained from all the participants. Viral RNA was extracted from plasma samples with Smi-test nucleic acid extraction kit (Genome Science, Fukushima, Japan). The HIV-1 RT gene was amplified by RT-PCR using One Step RNA PCR Kit (Takara Shuzo, Otsu, Japan). Nested PCR was conducted subsequently to amplify enough DNA for direct sequencing. The primer sets were T1 and T4 for the first PCR, and T12 and T15 for the second PCR (Hachiya et al., 2004). Specific precautions, including physical separation of processing areas, were taken to avoid template and amplified product carryover. Stringent quality control to prevent PCR contamination was employed to protect against cross-contamination of product DNA. Direct sequencing was performed using dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster, CA) and model 3700 automated DNA sequencer (Applied Biosystems). Amino acid sequences were deduced with Genetyx-Win program version 6.1 (Software Development, Tokyo).

Antiviral agents and cells

EFV, NVP, and 3TC were generously provided by Merck Co. Inc. (Rahway, NJ), Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, CT), and Nippon Glaxo-SmithKline (Tokyo, Japan), respectively. AZT and d4T were purchased from Sigma Co. (Tokyo, Japan). TDF was purchased from Moravek Biochemicals (Brea, CA). COS-7 cells and MT-2 cells were grown in Dulbecco's modified eagle medium (DMEM) and RPMI 1640, respectively, supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (0.1 mg/ml). MAGIC-5 cells (CCR5 and CD4 expressing HeLa-LTR-β-gal cells) (Hachiya et al., 2001, 2003, 2004) were grown in DMEM supplemented with 10% FCS, hygromycin B (0.1 mg/ml) (Invitrogen Co., San Diego, CA) and blasticidin (1000 ng/ml) (Funakoshi, Tokyo).

Generation of recombinant HIV-1 clones

The desired mutations were introduced into the *Xma*I–*Nhe*I region of pTZNX, which encodes Gly-15 to Ala-267 of HIV-1 RT (strain BH 10), by the oligonucleotide-based mutagenesis method (Hachiya et al., 2004; Kodama et al., 2001). The *Xma*I–*Nhe*I fragment was inserted into pNL_{H219Q}, which was modified from pNL101 and encoded the full genome of HIV-1. pNL_{H219Q} harbors H219Q mutation in HIV-1 Gag region, which facilitated HIV-1 replication in MT-2 cells (Gatanaga et al., 2002). Determination of the nucleotide sequences of plasmids confirmed that each clone had the desired mutations but no unintended mutations. Each molecular clone was transfected into COS-7 cells with GenePORTER Transfection Reagent (Gene Therapy Systems, San Diego, CA), and thus obtained virions were harvested 48 h after transfection and stored at −80 °C until use.

Induction of HIV-1 resistant to NNRTIs

The infectious HIV-1 clones harboring wild-type K103 ($HIV\text{-}1_{WT}$) or K103R ($HIV\text{-}1_{K103R}$) in RT were propagated in MT-2 cells in the presence of increasing concentrations of EFV and NVP, respectively (Gatanaga et al., 2002; Nunberg et al., 1991; Tanaka et al., 1997; Yoshimura et al., 1999). Briefly, MT-2 cells (1×10^5) were exposed to $HIV\text{-}1_{WT}$ or $HIV\text{-}1_{K103R}$ (500 blue cell-forming units [BFU] in MAGIC-5 cells) (Hachiya et al., 2001, 2003, 2004) and cultured in the presence of EFV and NVP at the initial concentrations of 3 nM and 30 nM, respectively. Viral replication was monitored by observation of the cytopathic effect in MT-2 cells. The culture supernatant was harvested on day 7 of culture and used to infect fresh MT-2 cells for the next round of culture. When the virus began to propagate in the presence of the drug, the drug concentration was increased. This selection was carried out for a total of 25 and 19 passages for cultures with EFV and NVP, respectively. Proviral DNAs from lysates of infected MT-2 cells from several passages were sequenced as indicated. Proviral HIV-1 RT gene was amplified and sequenced by using the primers T1 and T4. When necessary, molecular cloning was performed by using pT7 Blue T-Vector (Novagen, CA) and their sequences were analyzed.

Drug susceptibility assay with MAGIC-5 cells

Recombinant HIV-1 susceptibility to various RT inhibitors (RTIs) was determined in triplicate by using MAGIC-5 cells as described previously (Hachiya et al., 2001, 2003, 2004). Briefly, MAGIC-5 cells were infected with adjusted virus stock (300 BFU) in various concentrations of RTIs, cultured for 48 h, fixed, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (Takara Shuzo, Ohtsu, Japan). The blue-stained cells were counted under a light microscope. Drug concentrations that inhibited 50% of the stained cells of drug-free control (IC_{50}) were determined by referring to the dose–response curve.

Replication kinetic assay

MT-2 cells (10^5) were exposed to each infectious virus preparation (500 BFU) for 2 h, washed twice with PBS, and cultured in 1 ml of complete medium in the presence or absence of EFV or NVP. The culture supernatants were harvested every other day, and p24 Gag amounts were determined by chemiluminescence enzyme immunoassay (CLEIA) kit (Fuji-Rebio, Tokyo). Replication assays were performed in triplicate and repeated three times using independently generated virus preparations.

Competitive HIV-1 replication assay

Freshly prepared H9 cells (3 × 10^5) were exposed to the mixtures of paired virus preparations (300 BFU each) (HIV-1_{WT} vs. HIV-1_{K103R} in Figs. 4A–C, HIV-1_{WT} vs. HIV-1_{K103N} in Figs. 4D–F, and HIV-1_{WT} vs. HIV-$1_{K103R/V179D}$ in Figs. 4G–I) to be examined for their replication ability for 2 h, washed twice with PBS, and cultured in the absence (Figs. 4A, D, and G) or presence of EFV (3 nM for Fig. 4B, 10 nM for Figs. 4E and H) or NVP (30 nM for Fig. 4C, 100 nM for Figs. 4F and I) as describe previously (Hachiya et al., 2004; Kosalaraksa et al., 1999). On day 1, one-third of infected H9 cells were harvested and washed twice with PBS, and proviral DNAs were sequenced (0 week). Every 7 days, the supernatant of the virus culture was transmitted to new uninfected H9 cells, the cells harvested at the end of each passage (1, 2, and 3 weeks) were subjected to direct DNA sequencing of HIV-1 RT gene, and the viral population change was determined by the relative peak height on sequencing electrogram. The persistence of the original amino acid substitution was confirmed for all infectious clones used in this assay.

Acknowledgments

This work was supported in part by a Grant-in-Aid for AIDS research from the Ministry of Health, Labor, and Welfare of Japan (H15-AIDS-001), by the Program for the Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency (01–4), and by the Japan Antibiotics Research Association.

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