between our samples and an epidemic history proposed in the previous report [2]. Sixty-nine sequences were clustered with the cluster 2+3 in the previous report [2], which contained strains derived from IDUs in Vietnam. Six sequences formed a sub-cluster, which corresponded to the northern Vietnam/Guangxi IDU cluster (cluster 3). The remaining 11 sequences were paraphyly connected to the IDU cluster and were evolutionary related to heterosexual individuals in southern Vietnam and Thailand.

Notably, 34 out of 69 samples (49.3%) in the Vietnam IDU cluster were taken from IDU men, and 48 out of 69 were taken from men with HIV-negative wives, with considerable overlap of 25 samples. The remaining 11 samples contained seven from IDU men and six from men with HIV-negative wives. Co-receptor tropism was predicted in the 80 sequenced viruses: 9 could not be classified, 44 (55.0%) were deemed CXCR4 with the 5.75% cut-off by G2P. Of the 71 men with determined HIV tropism, 46 had HIV negative wives and 32 out of 46 (69.6%) had CXCR4-tropic strain. Additionally, 12 (48.0%) out of 25 men with HIV positive wives had CXCR4-tropic strain.

Follow up of HIV serodiscordant couples

Of the 101 serodiscordant couples enrolled in 2012, the team followed up 61 couples in 2014. In total, 126.5 person-years of observation were carried out (median observation period 24.1 months, range 13.1–34.6 months). All the men had been on ART. Follow-up HIV screening found no wives had seroconverted. The median frequency of sexual intercourse was 2 times per month during the observation year; 10 couples (16.4%) reported inconsistent condom use and 2 couples (3.3%) reported no condom use. Frequency of intercourse after HIV diagnosis as reported in the follow-up survey did not differ from the frequency reported in the initial survey, also post diagnosis (2 versus 2 times per month, $p = 0.868$). There was an increase in the percentage of men admitting not always using a condom during intercourse, from 5.6% to 16.4%, though in context the difference was not statistically significant ($p = 0.089$).

Discussion

High serodiscordance in northern Vietnam

This is the first study to investigate HIV-affected married couples in Vietnam with injecting drug use as a significant transmission route among men. To our surprise, HIV serodiscordance was as high as 62%, and particularly high among couples where the man's viral sequence belonged to the Vietnam IDU cluster (69.0%). This is despite the fact most couples had had a long duration of marriage with a considerable amount of unprotected sexual contact. This level of serodiscordance was notably higher than in previously published reports, including 24–31% from hospital studies in Thailand (where CRF01_AE is epidemic) and 42% and 55% from cross-sectional hospital studies in South India and Brazil, respectively [18–20].

As we analysed risk factors for HIV transmission among married couples, we found couples where the man had an IDU history still displayed a significantly high level of serodiscordance. To our knowledge, this association has not been previously demonstrated. It appears independent from sexual behaviour and other risk factors, such as genital ulcers and the use of ART. Although men with HIV positive wives had engaged in more unprotected sex after the HIV diagnosis of men than men with HIV negative wives, most wives were deemed to have seroconverted before the HIV diagnosed of men, and not during the duration between diagnosis of men and that of their wives. In most cases, the HIV diagnosis of wives was made immediately after the diagnosis of their husbands, and men had developed a more advanced clinical stage at the HIV diagnosis. Therefore, sexual behaviour after the HIV diagnosis of men was not deemed a risk factor in our setting. The above finding may indicate injecting drug use as a predominant

mode of blood-borne transmission may be linked to the high serodiscordance among Vietnamese couples. Furthermore, the phylogenetic analysis demonstrated the dominance of IDU-associated HIV strains among the study population, regardless of whether drug use was linked to transmission. This may explain why the proportion of HIV-positive wives was smaller even among men with only heterosexual transmission risk than among married men in northern Thailand, also with heterosexual contact as the main transmission risk [19]. The dominance of HIV serodiscordance or lower seroconversion rate among stable heterosexual couples in which the man was infected by parenteral transmission has been observed in other reports. For instance, the three primary types of HIV epidemic in China have been blood-donor centred in Henan, IDU centred in Yunnan, and sexual-transmission centred in other areas; of these the Henan and Yunnan epidemics have a lower seroconversion rate among HIV serodiscordant couples [21], though it should be noted the study did not systematically analyse HIV risk factors as well as transmission efficacy. A multi-centre cohort study of HIV-positive men with haemophilia in the United States reported only 13% seroconcordance [22]. Lower seroconcordance (higher discordance) among heterosexual couples in which the male partner had an IDU history was also reported in Thailand, though the study attributed this difference to the effect of subtype differences; IDU men were infected with subtype B whereas non-IDU men were infected with subtype CRF01_AE [18]. Taken with the results of our study, these findings suggest prior mode of transmission may contribute to subsequent transmission efficacy. Therefore we hypothesize that HIV strains derived from the IDU population in northern Vietnam transmit less efficiently through sexual contact than do other strains. On the other hand, an IDU-associated virus already transmitted through heterosexual contact may regain some of this transmission efficacy. Though it is difficult to speculate on mechanisms that might bring about this potential difference, some unique characteristics of HIV strains in Vietnam, such as the dominance of CXCR4 tropism observed in our study or a previous report [23], may have contributed to the apparent lower sexual transmission efficacy among stable couples in northern Vietnam. However, only half of the HIV *env* sequences could be successfully evaluated, and sequence data of the V3 loop alone cannot determine co-receptor tropism of CRF01_AE stains [24]. Therefore, there was insufficient data to discuss the relationship between viral factors and the transmission efficacy. In addition, there are several limitations in our study. First, behavioural data relies on self-reporting, making it difficult to avoid recall bias and social desirability bias. In this case, however, we believe our behavioural data is meaningful, as there was strong concordance between the desire to conceive and the frequency of sexual intercourse, as well as between answers obtained from men and the corresponding answers obtained from their wives. The second is the limited number of available viral load data due to infrastructural limitations. The third is the lack of virological confirmation regarding intra-couple transmission. The fourth is the risk factor analysis was done in the form of a cross-sectional design, meaning we could not discuss causality and were forced to exclude cases in which the man had died before the enrolment period. Taken together, unmeasured confounding factors might be underlying the results; the further investigation is required to discuss the possible risk factors among this population.

There are other potential influences that should be taken into consideration. One concerns host factors; CCR5 mutation, high secretion of beta chemokines, or killer immunoglobulin-like receptor (KIR)/HLA combination are well known to influence HIV transmission [25]. Although CCR5 delta 32 mutation is rare among Asian populations, two possible protective CCR5 mutations were reported in highly exposed persistent seronegative (HEPS) Vietnamese IDUs [26]. High Natural Killer (NK) cell activity [27], enhanced CD4+ T cell response to beta chemokines, and inhibition of post-entry viral replication [28] were also observed in these groups. Some wives in the study may also harbour these protective genetic factors, and this

should be considered when trying to explain the relative lack of HIV transmission. However, as the study population was drawn from a single ethnic group with relatively uniform genetic distribution, host factors such as these would be unlikely to account for the full lack of HIV transmission efficacy. Another influence concerns risk behaviours of wives. Wives reported few risk behaviours save for those associated with having HIV positive husbands. Although a small number of wives tested positive for hepatitis B and/or hepatitis C co-infection implying the presence of past risk behaviours, the overall impact would likely not be considerable. The result of the survey of wives is also consistent with a published review of the HIV epidemic among Vietnamese women [9]. Therefore among our study population, the behaviour of wives would be highly unlikely to account for any meaningful difference.

Advanced HIV infection at the time of diagnosis

Among the study population, HIV diagnosis in the advanced stage was negatively associated with HIV transmission to wives. This finding is compatible with that of a previously published hospital-based study in Thailand [19], though it does contradict the widely accepted understanding that viral load plays a key role in HIV transmission as outlined in several large population based studies [29,30]. We believe this discrepancy can be attributed in part to the hospital-based setting. Our study and the study in Thailand were both conducted at referral hospitals where most patients were symptomatic with a low CD4+ T cell count and with HIV-negative spouses that had been exposed to the virus for a substantial amount of time. It is also plausible that men at an advanced stage of infection had less sexual intercourse with their wives at this time because they were feeling unwell, and this could explain the negative association with HIV transmission. However, there was no difference in sexual behaviors according to clinical status of men at the baseline.

Co-infection with HBV

Interestingly, there was a significant positive association between HBV co-infection among index men and HIV transmission to wives. Although there have been reports showing high mortality among HIV-positive patients with HBV co-infection in Thailand [31] and the United States [32], to our knowledge HBV co-infection has not been found to enhance the risk of HIV sexual transmission. According to a previously published in vitro study, HBV-X protein super-induces on-going HIV replication and HIV-1 long-terminal repeat transcription [33]. Although no studies known to us have shown HBV co-infection to increase the HIV viral load, HIV replication in the genital area may have been further activated in HBV-co-infected men, and this in turn may have facilitated transmission.

Lack of seroconversion in the follow-up study

We found no seroconversion after the median 24.1 months of follow-up, despite some degree of unprotected intercourse and a downward tendency in condom use. This was in contrast to the results of studies conducted in the pre-ART era, which showed a substantial seroconversion rate (3.0–12.0/100 person-years) [19,34–37]. This difference is likely explained by high ART coverage reducing the transmission risk from index men [30], as well as better condom use. Although substance abuse is considered related to poor treatment adherence [38], our results imply proper education and treatment can lead to successful prevention of sexual transmission in long-term relationships where substance abuse is an issue. Diagnosing HIV in the early stages and providing counselling and treatment is now the cornerstone of the HIV prevention effort. Our results endorse the importance of screening both partners making up HIV affected couples and actively introducing early ART interventions.

Conclusions

This is the first study comprehensively investigating HIV-affected married couples in Vietnam. The proportion of HIV serodiscordance was high, particularly among couples where the HIV-infected man had an IDU history, and in both cases despite substantial sexual risk behaviours before the diagnosis. A relatively high number of at-risk wives therefore remain HIV-negative and can still be protected if couples are made aware of this serodiscordance through screening. As our study is likely indicative of the wider population, such screening could lead to the prevention of eventual transmission to wives. Longitudinal follow-up demonstrated no seroconversion among HIV-serodiscordant couples where HIV-positive men had received ART. Taken as a whole, our study re-emphasizes the importance of active HIV screening and early ART provision to couples.

Supporting Information

S1 Fig. Evolutionary relationships of gp120 sequences from 80 men. The phylogeny was inferred using the Maximum Likelihood method and the tree with the highest log likelihood (-28676.0757) is shown. Sequences belonging to other subtype than CRF01_AE were omitted after the inference and represented a branch connected to the tree-root. Bootstrap probability with >80% was indicated for each cluster. Red open square, red open circle, and red circle indicated virus samples from heterosexual men, IDU men, and men with both risk in our subjects, respectively. Black square, black open circle, and black circle showed reference sequences in the previous report [2] belonging to cluster 1 (southern Vietnam heterosexual individuals), cluster 2 (southern Vietnam IDU individuals), and cluster 3 (northern Vietnam or Guangxi IDU individuals), respectively. Dotted line highlights CRF01_AE references retrieved from HIV sequence database. Grey and brown hatching showed the sample clusters corresponding to cluster 2 and 3, respectively.
(PDF)

Acknowledgments

The authors acknowledge the kindness and contributions of all the couples participating in the study, as well as the efforts of the staff at the HIV outpatient clinic of Bach Mai Hospital, in facilitating the investigation. We offer further thanks to Keiko Saito and Nguyen Thi Huyen for their work in setting up the Couple Cohort study. Lastly we are grateful to Dr. Koen Peeters Grietens at the Institute of Tropical Medicine in Antwerp for his critical review of our data.

Author Contributions

Conceived and designed the experiments: IS JT NT LMY KA SO. Performed the experiments: IS CDD TTD QPL LATN TVTV TQN TTTP. Analyzed the data: IS JT QPL LATN TS LMY KA SO. Contributed reagents/materials/analysis tools: IS JT QPL LATN TS LMY KA SO. Wrote the paper: IS JT CDD TTD QPL LATN TVTV TQN NT TS LMY TTTP KA SO.

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Different Effects of Nonnucleoside Reverse Transcriptase Inhibitor Resistance Mutations on Cytotoxic T Lymphocyte Recognition between HIV-1 Subtype B and Subtype A/E Infections

Nozomi Kuse,[a] Mohammad Arif Rahman,[a] Hayato Murakoshi,[a] Giang Van Tran,[a] Takayuki Chikata,[a] Madoka Koyanagi,[a] Kinh Van Nguyen,[c] Hiroyuki Gatanaga,[a,d] Shinichi Oka,[a,d] Masafumi Takiguchi[a,b]

Center for AIDS Research[a] and International Research Center for Medical Sciences,[b] Kumamoto University, Kumamoto, Japan; National Hospital of Tropical Diseases, Dong Da District, Hanoi, Vietnam[c]; AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan[d]

ABSTRACT

The effect of antiretroviral drug resistance mutations on cytotoxic T lymphocyte (CTL) recognition has been analyzed in HIV-1 subtype B infections, but it remains unclear in infections by other HIV-1 subtypes that are epidemic in countries where antiretroviral drugs are not effectively used. We investigated the effect of nonnucleoside reverse transcriptase (RT) inhibitor (NNRTI)-resistance mutations (Y181C, Y181I, and Y181V) on epitope recognition by CTLs specific for 3 different HIV-1 epitopes (HLA-A*02:01-restricted IV10, HLA-B*35:01-restricted NY9, and HLA-C*12:02-restricted KY9) in subtype B and subtype A/E infections and the accumulation of these mutations in treatment-naive Japanese and Vietnamese. These NNRTI-resistance mutations critically affected NY9-specific and KY9-specific T cell responses in the subtype B infections, whereas they showed a different effect on IV10-specific T cell responses among the subtype B-infected individuals. These mutations affected IV10-specific T cell responses but weakly affected NY9-specific T cell responses in the subtype A/E infections. The substitution at position 3 of NY9 epitope which was found in the subtype A/E virus differently influenced the peptide binding to HLA-B*35:01, suggesting that the differences in peptide binding may result in the differences in T cell recognition between the subtype B virus and A/E virus infections. The Y181C mutation was found to be accumulating in treatment-naive Vietnamese infected with the subtype A/E virus. The present study demonstrated different effects of NNRTI-resistance RT181 mutations on CTL responses between the 2 subtype infections. The Y181C mutation may influence HIV-1 control by the CTLs in Vietnam, since this mutation has been accumulating in treatment-naive Vietnamese.

IMPORTANCE

Antiretroviral therapy leads to the emergence of drug-resistant HIV-1, resulting in virological and clinical failures. Though HIV-1-specific CTLs play a critical role in HIV-1 infection, some of drug resistance mutations located in CTL epitopes are known to affect HIV-1-specific CTL responses. Nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistance RT181 mutations are frequently observed in patients treated with NNRTIs. Such drug resistance mutations may have an influence on immune control by HIV-1-specific CTLs, especially in countries where antiretroviral drugs are not effectively used. We here investigated the effect of three NNRTI-resistance RT181 mutations on immune responses by HIV-1-specific CTLs and the recent accumulation of these mutations in treatment-naive Vietnamese infected with HIV-1 subtype A/E virus. RT181 mutations affected CTL recognition in both subtype A/E and B infections, while the RT Y181C mutation has been accumulating in treatment-naive Vietnamese. The results suggest that the Y181C mutation may influence HIV-1 control by CTLs in Vietnam.

Drug resistance is a major obstacle to effective and long-term antiretroviral therapy (ART) for treatment of HIV-1 infections. Mutations conferring resistance to antiretroviral drugs cluster within the viral protein targeted by antiretroviral drugs, such as nucleoside reverse transcriptase (RT) inhibitors (NRTIs), nonnucleoside RT inhibitors (NNRTIs), protease inhibitors (PIs), and integrase inhibitors (INIs). Some of such drug resistance mutations are located in epitopes recognized by HIV-1-specific cytotoxic T lymphocytes (CTLs). Although CTLs have a critical role in the control of HIV-1 replication during acute and chronic phases of HIV-1 infections (1–4), escape mutants, mostly selected by HIV-1-specific CTLs, could result in the loss of immune control (4–8). It is well known that some drug resistance mutations also influence immune recognition (9–12).

Some drug resistance mutations have been shown to affect CTL recognition. PI-resistance mutations M46I, I47A, G48V, and I50V as well as INI-resistance mutation E92Q impair or abrogate

Received 14 April 2015 Accepted 4 May 2015
Accepted manuscript posted online 13 May 2015
Citation Kuse N, Rahman MA, Murakoshi H, Tran GV, Chikata T, Koyanagi M, Nguyen KV, Gatanaga H, Oka S, Takiguchi M. 2015. Different effects of nonnucleoside reverse transcriptase inhibitor resistance mutations on cytotoxic T lymphocyte recognition between HIV-1 subtype B and subtype A/E infections. J Virol 89:7363–7372. doi:10.1128/JVI.00974-15.
Editor: G. Silvestri
Address correspondence to Masafumi Takiguchi, masafumi@kumamoto-u.ac.jp.
N.K., M.A.R., and H.M. contributed equally to this article.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.00974-15.

doi:10.1128/JVI.00974-15

CTL recognition (11, 12), whereas NRTI-resistance mutations such as M41L, L74V, M184V, and T215Y/F do not impair CTL recognition (9). HLA-A*02:01-restricted YV9-specific $CD8^+$ T cells effectively recognize the M184V mutant epitope and inhibit the replication of M184V mutant viruses *in vitro* (13). Interestingly, individuals with the $CD8^+$ T cell responses to this mutation have a significantly lower plasma viral load than those without the $CD8^+$ T cell responses to it (13). K103N and Y181C mutations were frequently observed in patients treated with NNRTIs such as efavirenz (EFV) and nevirapine (NVP) (14). CTLs directed against K103N are not known, whereas the Y181C mutation was reported to be located in several CTL epitopes restricted by various HLA molecules (15). However, the effect of the Y181C mutation on these epitope-specific CTLs still remains unclear.

Y181I and Y181V are known as major NNRTI-resistance mutations in addition to the Y181C mutation. The Y181C mutation is selected in patients receiving NVP, etravirine (ETR), and ripivirine (RPV) and reduces susceptibility to these drugs and EFV, whereas Y181I/V mutations are selected by NVP and ETR and cause resistance to these drugs and to RPV (16–23). These 3 RT181 mutations are located in previously reported epitopes, HLA-A*02:01-restricted IV10 (RT 180–189), HLA-B*35:01-restricted NY9 (RT 175–183), and HLA-C*12:02-restricted KY9 (RT 173–181), in HIV-1 subtype B (24–26). We investigated here the effect of the RT181 mutations on epitope recognition by CTLs specific for 3 different epitopes in HIV-1 subtype B and subtype A/E infections and the accumulation of these mutations in treatment-naive Japanese and Vietnamese to clarify the effect of these NNRTI-resistance mutations on immune control of HIV-1.

MATERIALS AND METHODS

Samples from HIV-1-infected individuals. This study was approved by the Ethical Committee of Kumamoto University and the National Center for Global Health and Medicine, Japan, and National Hospital for Tropical Diseases, Vietnam. Written informed consent was obtained from all subjects according to the Declaration of Helsinki. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from whole blood. HIV-1-infected, antiretroviral-naive individuals were recruited for the present study.

Cells. C1R-A*0201, -B*3501, and -C*1202, 721.221-CD4-B*3501 and -C*1202, and RMA-S-B*3501 were previously generated (25–29). 721.221-CD4-A*0201 and RMA-S-A*0201 were obtained by transfecting 721.221-CD4 cells and RMA-S cells, respectively, with the *HLA-A*02:01* gene. RMA-S-C*1202 cells were generated by transfecting RMA-S cells with the *HLA-C*12:02* gene. These cells were maintained in RPMI 1640 medium containing 5% fetal bovine serum (FBS) and 0.15 mg/ml hygromycin B.

HIV-1 clones. An infectious proviral clone of HIV-1, pNL-432, was previously reported (30). NL-432-Y181C, -Y181V, and -Y181I mutant viruses were generated by introducing the Y181C, Y181V, and Y181I mutations, respectively, into NL-432 by using site-directed mutagenesis (Invitrogen) based on overlap extension. NL-432-$_{NY9\text{-}3E}$, NL-432-$_{NY9\text{-}3E}$-Y181C, NL-432-$_{NY9\text{-}3E}$-Y181V, and NL-432-$_{NY9\text{-}3E}$-Y181I mutant viruses were generated by introducing the NY9-3E mutation into NL-432, NL-432-Y181C, -Y181V, and -Y181I viruses, respectively, by using site-directed mutagenesis based on overlap extension.

Induction of HIV-1 epitope-specific bulk T cells. PBMCs from HIV-1-infected individuals were stimulated with wild-type (WT) peptide (100 nM) and then cultured in RPMI 1640 medium containing 10% fetal bovine serum (R10) and 200 U/ml human recombinant interleukin-2 (rIL-2). After about 14 days, these bulk-cultured T cells were used for the ^{51}Cr release assay or intracellular cytokine staining (ICS) assay.

Generation of HIV-1 epitope-specific CTL clones. HIV-1 epitope-specific CTL clones were generated from bulk-cultured T cells by limiting dilution. Each well contained 200 μl of cloning mixture (1×10^6 irradiated PBMCs and 1×10^5 irradiated C1R cells expressing HLA pulsed with the peptide in R10 containing 200 U/ml human rIL-2 and 2.5% phytohemagglutinin soup).

^{51}Cr release assay. The cytotoxic activity of T cells toward target cells pulsed with peptide or those infected with HIV-1 was measured as described previously (31). Target cells (2×10^3/well) labeled with $Na_2{}^{51}CrO_4$ were cocultured with effector cells for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the counts per minute (cpm) in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr release was determined by measuring the counts per minute in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was defined as follows: (cpm exp − cpm spn)/(cpm max − cpm spn) × 100 (where "cpm exp" is the counts per minute in the supernatant in the wells containing both target and effector cells). Relative specific lysis of peptide-pulsed target cells was defined as follows: (specific lysis of exp − specific lysis of cells without peptide). Infected cells were used as target cells when approximately 50% to 70% of the total cells were p24 Ag-positive cells. Relative specific lysis of target cells infected with HIV-1 was defined as follows: (specific lysis of exp − specific lysis of uninfected cells)/(frequency of p24 antigen-positive cells among infected cells).

ICS assay. C1R cells prepulsed with the peptide for 1 h at 37°C were cocultured with T cells in the presence of brefeldin A (10 μg/ml) at 37°C for 4 h. Subsequently, the cells were stained with phycoerythrin (PE)-labeled anti-CD8 monoclonal antibody (MAb) at 4°C for 30 min, fixed with paraformaldehyde solution at 4°C for 20 min, and then permeabilized with permeabilization buffer (0.1% saponin–10% FBS–phosphate-buffered saline [PBS]) at 4°C for 10 min. Thereafter, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-gamma interferon (IFN-γ) MAb at room temperature for 20 min and then washed twice with the permeabilization buffer. The percentage of $CD8^+$ cells producing IFN-γ was analyzed by flow cytometry (FACSCanto II).

Enzyme-linked immunosorbent (ELISPOT) assay. The peptides and PBMCs were added to a plate precoated with 5 mg/ml anti-IFN-γ MAb 1-D1K. The cells were subsequently incubated for 16 h at 37°C in 5% CO_2 and then washed with PBS. Biotinylated anti-IFN-γ MAb was added at 1 mg/ml, and the cells were incubated at room temperature for 90 min and subsequently incubated with streptavidin-conjugated alkaline phosphatase at room temperature for 60 min. Individual cytokine-producing cells were detected as dark spots after a 20-min reaction with 5-bromo-4-chloro-3-idolyl phosphate and nitro blue tetrazolium by using an alkaline phosphatase-conjugate substrate. The spot number was counted by using an Eliphoto counter. The $CD8^+$ T cells without peptide stimulation were used as a negative control.

HLA stabilization assay. Peptide binding activity for HLA was examined by performing an HLA stabilization assay using RMA-S cells as described previously (29). Cells were incubated at 26°C for 17 h and then pulsed with peptides at 26°C for 1 h. After incubation for 3 h at 37°C, the cells were stained with SFR8-B6 antibody (used for HLA-B*35:01 and -C*12:02) or BB7.2 antibody (used for HLA-A*02:01) and with FITC-conjugated sheep anti-mouse IgG. The mean fluorescence intensity (MFI) was measured by flow cytometry (FACSCanto II). The concentration of peptide giving the half-maximal binding level (BL_{50}) in the staining results was calculated.

Sequencing of plasma RNA. Viral RNA was extracted from the plasma of HIV-1-infected individuals by using a QIAamp MinElute Virus Spin kit (Qiagen). cDNA was synthesized from the RNA with a SuperScript III first-strand synthesis system for RT-PCR and a random primer (Invitrogen). The HIV-1 polymerase (Pol) gene was amplified by nested PCR using *Taq* polymerase (Promega). Sequencing reactions were performed

TABLE 1 Known CTL epitopes incorporating RT181 mutations induced by NNRTI drugs

HBX2 position (drug resistance mutation Y181C/V/I)	Subtype	Consensus sequence[a]	Epitope	HLA restriction	No. of individuals with viruses with indicated consensus sequence/total no. of individuals (%)	
					Los Alamos database	Our patient cohort
RT180–189	B	IYQYMDDLYV	IV10	A*02:01	1,171/1,293 (90.6)	319/363 (87.9)
	A/E	-Y--------	IV10		341/366 (93.2)	370/377 (98.1)
RT175–183	B	NPDIVIYQY	NY9	B*35:01	722/1,293 (55.8)	173/363 (47.7)
	A/E	--E---Y--	NY9-3E		128/366 (35.0)	179/377 (47.5)
RT173–181	B	KQNPDIVIY	KY9	C*12:02	649/1,293 (50.2)	155/363 (42.6)
	A/E	IK--E---Y	Not epitope		87/366 (23.8)	112/377 (29.7)

[a] Boldface characters indicate RT181 position.

with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and analyzed by use of an ABI 3500 genetic analyzer.

RESULTS

NNRTI-resistance mutations at RT181 are located on 3 different CTL epitopes. RT181 is located at positions 2, 7, and 9 of 3 known subtype B-derived CTL epitopes, HLA-A*02:01-restricted IV10, HLA-B*35:01-restricted NY9, and HLA-C*12:02-restricted KY9, respectively. We first analyzed the consensus sequence of these epitopes in chronically HIV-1 subtype B-infected Japanese and subtype A/E-infected Vietnamese individuals (Table 1). The consensus sequence of IV10 (IYQYMDDLYV) was found at a frequency of approximately 90% in both subtype B and subtype A/E viruses, whereas NY9 was a variable epitope, and its consensus sequences were different between subtype B virus and subtype A/E virus (NPDIVIYQY and NPEIVIYQY, respectively). These consensus sequences were found at frequencies of 40% to 50% in the subtype B and A/E viruses. The sequence for the KY9 epitope in the subtype A/E virus was IKNPEIVIY, but the specific CTLs were not induced in 7 HLA-C*12:02$^+$ Japanese infected with the subtype A/E virus (data not shown). KY9 was also a variable epitope, and its consensus sequence (KQNPDIVIY) was found in approximately 50% of the subtype B viruses.

Effect of RT181 mutations on epitope recognition by HLA-A*02:01-restricted IV10-specific and HLA-C*12:02-restricted KY9-specific CTLs. To investigate the effect of the RT181 mutations on epitope recognition by CTLs specific for these epitopes, we first analyzed HLA-A*02:01-restricted IV10-specific CTLs. We analyzed 7 and 14 HLA-A*02:01$^+$ individuals chronically infected with the subtype B virus and the subtype A/E virus, respectively. IV10-specific T cells were detected in 4 individuals infected with the subtype B virus and in 3 with the subtype A/E virus. We analyzed the responses of IV10-specific T cells from these patients to 3 different mutant peptides by performing the ELISPOT assay using the peptides (Fig. 1A). No responses of the T cells to these mutations were found in 3 subtype B-infected (KI-691, KI-775, and KI-840) and 3 subtype A/E-infected (KI-837, VI-080, and VI-087) individuals, whereas the responses to the 3 mutant peptides were detected in one individual (KI-634) infected with the subtype B virus. To confirm these findings, we established IV10-specific bulk T cells from individuals infected with the subtype B virus or the subtype A/E virus and then tested these T cell responses to mutant peptides by performing a ^{51}Cr release assay. The IV10-specific bulk T cells which were established from these individuals, who did not show cross-responses to the mutant peptides in the ELISPOT assay, did not cross-recognize these mutant peptides at concentrations of lower than 10 nM, whereas the bulk T cells from KI-634 exhibited cross-responses to the mutant peptides (Fig. 1B). These mutant peptides showed higher affinity for HLA-A*02:01 than did the wild-type peptides (Fig. 1C) (BL_{50} of 681.4 ± 87.6 μM for the WT versus 85.4 ± 6.8 μM for 2C [$P < 0.01$], 32.6 ± 12.0 μM for 2V [$P < 0.01$], and 30.2 ± 2.3 μM for 2I [$P < 0.01$]), suggesting that these mutations partially affected the epitope recognition by T cell receptors (TCRs) on the IV10-specific T cells from KI-634 but critically affected that by TCRs on the T cells from other individuals. We further investigated the effect of RT181 drug resistance mutations on the abilities of CTLs to recognize target cells infected with HIV-1. IV10-specific bulk T cells from KI-775 and KI-837 recognized NL-432 WT-infected target cells but not NL-432-Y181C, -Y181V, or -Y181I-infected ones (Fig. 1D). These results, taken together, indicate that all 3 mutations affected epitope recognition by IV10-specific CTLs from the 2 individuals (KI-775 and KI-837). In contrast, the bulk T cells from KI-634 exhibited similar killing activities for the target cells infected with WT virus and those infected with NL-432-Y181C virus but failed to kill the target cells infected with NL-432-Y181V or with NL-432-Y181I (Fig. 1D). Together, these findings suggest that the V and I mutations affected presentation of the epitope and recognition of TCR from KI-775 and KI-837 but that the C mutant epitope was effectively presented in HIV-1-infected cells but reduced the recognition of TCR from KI-775 and KI-837. Thus, the RT181 mutations mostly affected epitope recognition by the HLA-A*02:01-restricted CTLs in HIV-1 subtype B and subtype A/E infections.

We next analyzed HLA-C*12:02-restricted KY9-specific T cell responses to these 3 mutations. By performing an ELISPOT assay, we found HLA-C*12:02-restricted KY9-specific T cells in only 3 of 19 subtype B-infected HLA-C*12:02$^+$ individuals. The T cells from 2 representative cases were analyzed for 3 mutant peptides by ELISPOT assay. The T cells from KI-108 showed significantly weaker responses to 9C or 9V mutant peptide than to wild-type peptide, whereas those from KI-504 revealed significantly weaker responses to 9C peptide than to wild-type peptide (Fig. 2A). These results indicate that 9C critically affected recognition of KY9-specific T cells. Two HLA-C*12:02-restricted KY9-specific CTL clones established from KI-108 failed to recognize the mutant peptides at concentrations of from 0.01 to 10 nM (Fig. 2B). These

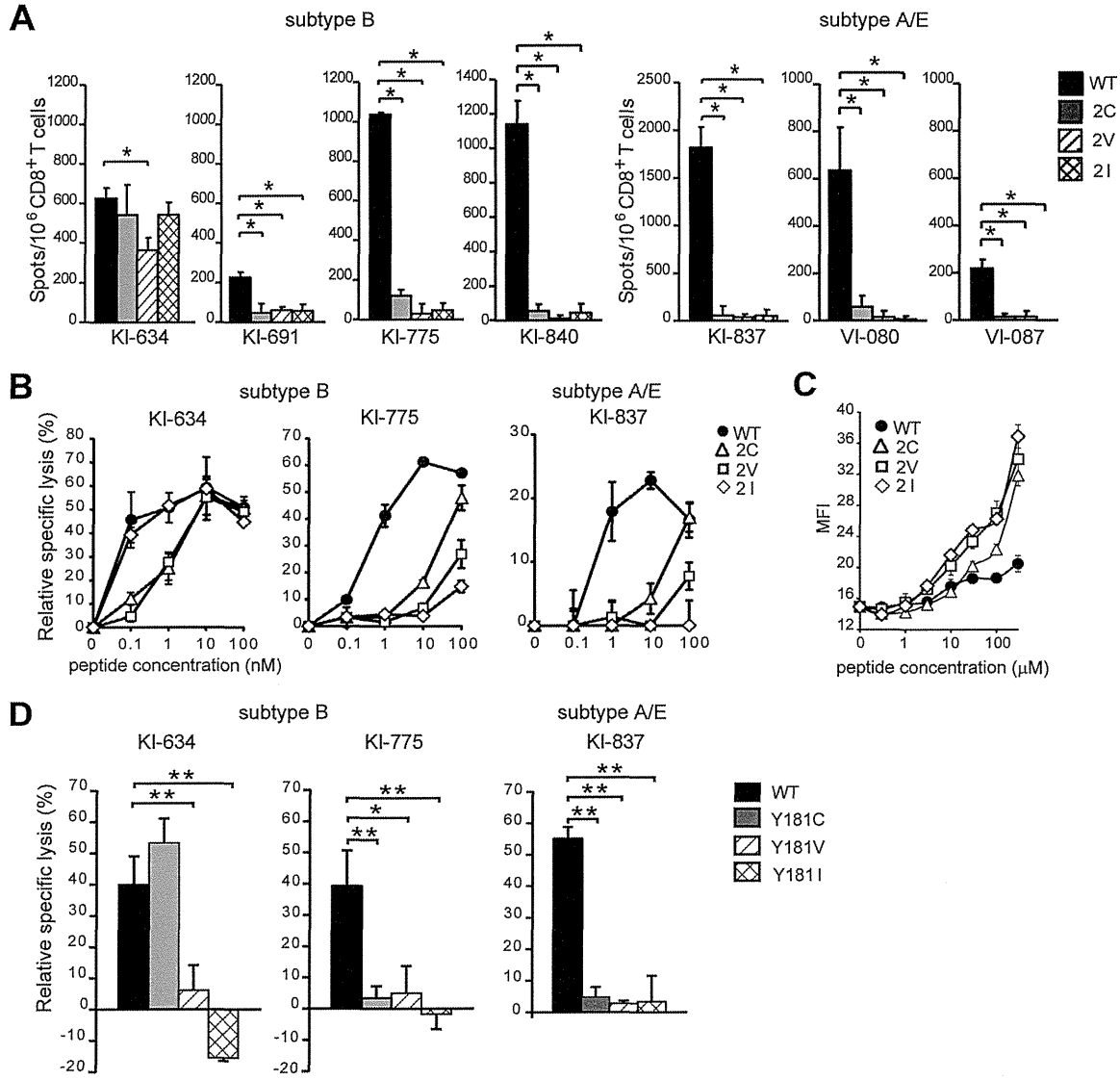

FIG 1 Recognition of RT181 mutations by HLA-A*02:01-restricted IV10-specific CTLs. (A) *Ex vivo* $CD8^+$ T cell responses to IV10 (WT) or the mutant peptides in HLA-A*02:01$^+$ individuals infected with the subtype B virus or the subtype A/E virus. $CD8^+$ T cell responses to IV10 or the mutant peptides by PBMCs in 7 HIV-1-infected individuals were analyzed by performing the ELISPOT assay using a 100 nM concentration of peptides. (B) Relative cytotoxic activity of HLA-A*02:01-restricted IV10-specific bulk T cells toward C1R-A*0201 cells prepulsed with the peptides. It was measured by performing the ^{51}Cr release assay. (C) Binding of the IV10 and mutant peptides to HLA-A*02:01. Binding ability was measured by performing the HLA class I stabilization assay using RMA-S-A*0201 cells. (D) Relative cytotoxic activity of IV10-specific bulk T cells against 721.221-CD4-HLA-A*0201 cells infected with the wild-type (NL-432) or mutant viruses. The frequencies of p24 antigen-positive cells among 721.221-CD4-HLA-A*0201 cells infected with NL-432, NL-432-Y181C, NL-432-Y181V, and NL-432-Y181I were 62%, 44%, 51%, and 41%, respectively, for KI-634; 59%, 59%, 53%, and 41%, respectively, for KI-775; and 63%, 61%, 63%, and 60%, respectively, for KI-837. All data are shown as the means and ± standard deviations (SD) of the results of triplicate assays. Statistical analysis was performed by using the *t* test. *, $P < 0.05$; **, $P < 0.01$ (as indicated by the brackets).

mutations reduced the peptide binding to HLA-C*12:02 (Fig. 2C) (BL_{50} of 1.0 ± 0.1 μM for the WT versus 28,353.5 ± 8,088.9 μM for 9C [$P < 0.05$], 11.1 ± 1.2 μM for 9V [$P < 0.01$], and 76.1 ± 1.2 μM for 9I [$P < 0.01$]). Together, these results indicate that the failure of T cells to recognize the mutant peptide-pulsed cells resulted from a critical reduction in the binding of the mutant peptides to HLA-C*12:02. These T cell clones also failed to recognize the cells infected with these mutant viruses (Fig. 2D), suggesting that the Y181C, Y181I, and Y181V mutations affected control of these mutant viruses by KY9-specific CTLs in the subtype B infection.

Different effects of RT181 drug mutations on HLA-B*35:01-restricted NY9-specific CTL recognition between the subtype B and subtype A/E infections. We next investigated the effect of RT181 drug mutations on HLA-B*35:01-restricted NY9 (NPDIV IYQY)-specific T cells. First, we analyzed the responses of the NY9-specific T cells to the 3 mutant peptides (NY9-7C, NY9-7V, and NY9-7I) by use of the ELISPOT assay. We found NY9-specific T cell responses in 3 of 7 HIV-1 subtype B-infected HLA-B*35:01$^+$ individuals. The T cells responded much better to the wild-type peptide than to the NY9-7C, NY9-7V, or NY9-7I mutant peptide in these individuals (Fig. 3A). Indeed, in the ^{51}Cr release assay,

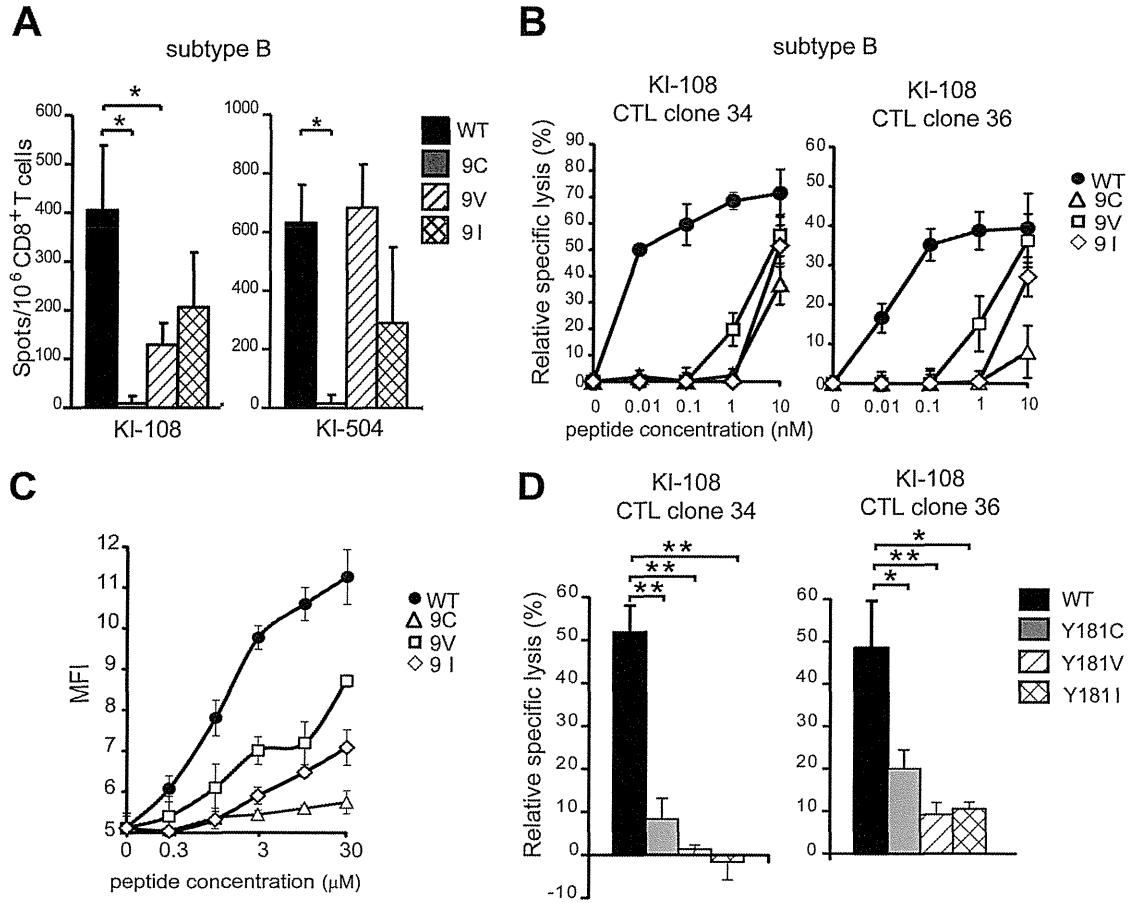

FIG 2 Recognition of RT181 mutations by HLA-C*12:02-restricted KY9-specific CTLs. (A) *Ex vivo* $CD8^+$ T cell responses to KY9 (WT) or the mutant peptides in HLA-C*12:02$^+$ individuals infected with subtype B virus. $CD8^+$ T cell responses to KY9 or the mutant peptides by PBMCs from 2 HIV-1-infected individuals were analyzed by performing the ELISPOT assay using a 100 nM concentration of the peptides. (B) Relative cytotoxic activity of HLA-C*12:02-restricted KY9-specific CTL clones toward C1R-C*1202 cells prepulsed with KY9 or the mutant peptides. (C) Binding of the KY9 and mutant peptides to HLA-C*12:02. Binding ability was measured by performing the HLA class I stabilization assay using RMA-S-C*1202 cells. (D) Relative cytotoxic activity of KY9-specific CTL clones against 721.221-CD4-HLA-C*1202 cells infected with the wild-type (NL-432) or mutant viruses. The frequencies of p24 antigen-positive cells among 721.221-CD4-HLA-C*1202 cells infected with NL-432, NL-432-Y181C, NL-432-Y181V, and NL-432-Y181I were 47%, 44%, 42%, and 54%, respectively. All data are shown as the means and ± SD of the results of triplicate assays. Statistical analysis was performed by using the *t* test. *, $P < 0.05$; **, $P < 0.01$ (as indicated by the brackets).

CTL clone 14-503 from an HLA-B*35:01$^+$ individual failed to recognize NY9-7C, NY9-7V, and NY9-7I mutant peptides, although this clone effectively recognized the WT peptide (Fig. 3B). The affinity of these mutant peptides for HLA-B*35:01 was slightly lower than that of the WT peptide (Fig. 3C) (BL_{50} of 0.8 ± 0.1 μM for the WT versus 1.6 ± 0.1 μM for 7C [$P < 0.01$], 1.0 ± 0.4 μM for 7V [$P = 0.44$], and 1.7 ± 0.2 μM for 7I [$P < 0.01$]), indicating that these mutations affected epitope recognition by TCRs on the NY9-specific T cells. The clone also failed to recognize target cells infected with NL-432-Y181C, -Y181V, or -Y181I (Fig. 3D). These results indicate that these mutations critically affected the epitope recognition by NY9-specific T cells in an HIV-1 subtype B infection.

On the other hand, the consensus sequence of this epitope in the subtype A/E virus (NY9-3E [NPEIVIYQY]) was different at position 3 (underlined) from the consensus sequence of that in the subtype B virus (Table 1). We first analyzed T cell responses to the NY9-3E peptide in HLA-B*35:01$^+$ Japanese individuals infected with the subtype A/E virus by performing the ELISPOT assay and found NY9-3E-specific T cell responses in 3 of 5 HLA-B*35:01$^+$ Japanese individuals infected with the subtype A/E virus (KI-667, KI-805, and KI-1124; Fig. 4A). Thus, NY9-3E was also recognized as a T cell epitope in the subtype A/E infection. We further analyzed the response of these T cells to the 3 mutant peptides (NY9-3E7C, NY9-3E7V, and NY9-3E7I). These T cells cross-recognized the 3 mutant peptides in both individuals (Fig. 4A). We next established HLA-B*35:01-restricted NY9-3E-specific bulk T cells from KI-667 and KI-805 by stimulating their PBMCs with NY9-3E peptide and then tested the recognition of these 3 mutant peptides by the T cells. These T cells showed a recognition pattern for these mutant peptides that was different from that seen with a subtype B-derived NY9-specific CTL clone. NY9-3E-specific bulk T cells from KI-667 similarly recognized wild-type and NY9-3E7I mutant peptides, but recognition of NY9-3E7C and NY9-3E7V mutant peptides was weaker than recognition of the wild-type one (Fig. 4B, left). In contrast, NY9-3E-specific bulk T cells established from KI-805 recognized NY9-3E wild-type and NY9-3E7C mutant peptides similarly and recognized NY9-3E7V and -3E7I mu-

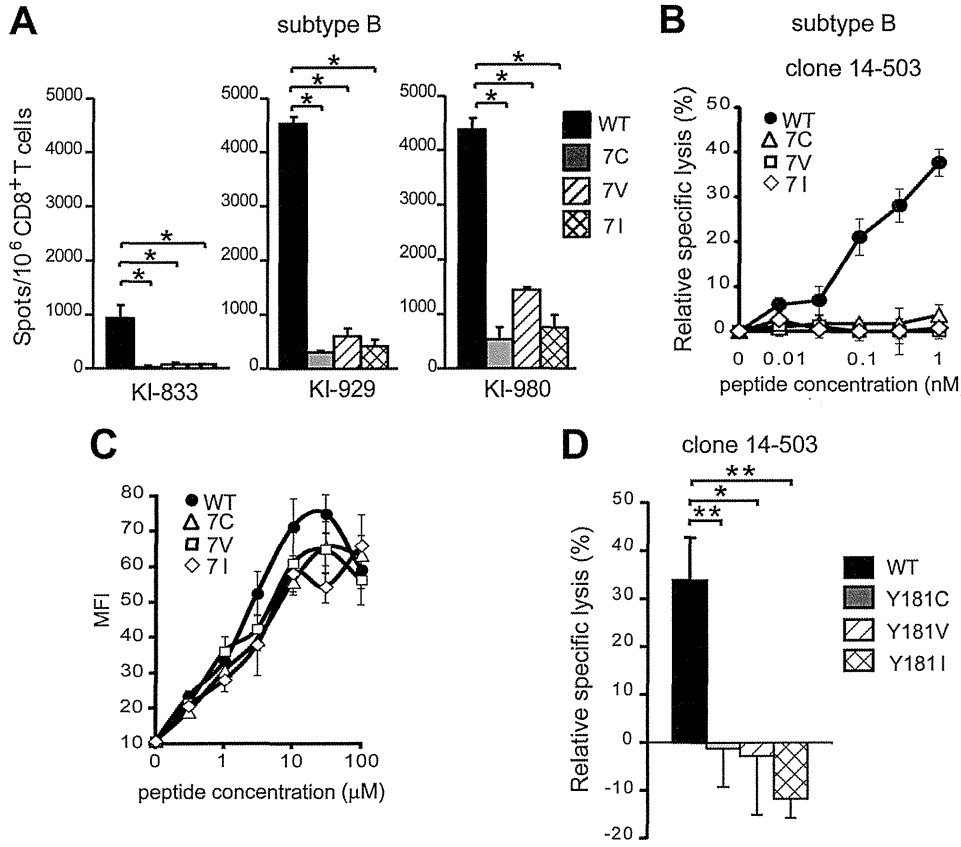

FIG 3 Effect of RT181 mutations on HLA-B*35:01-restricted NY9-specific CTL recognition in subtype B infections. (A) *Ex vivo* $CD8^+$ T cell responses to NY9 (WT) or the mutant peptides in HLA-B*35:01^+ individuals infected with subtype B virus. $CD8^+$ T cell responses to NY9 or the mutant peptides in PBMCs from 3 HIV-1 subtype B-infected individuals were analyzed by performing the ELISPOT assay using a 100 nM concentration of the peptides. (B) Relative cytotoxic activity of HLA-B*35:01-restricted NY9-specific CTL clones toward C1R-B*3501 cells prepulsed with WT or the mutant peptides. (C) Binding of the NY9 and mutant peptides to HLA-B*35:01. Binding ability was measured by performing the HLA class I stabilization assay using RMA-S-B*3501 cells. (D) Relative cytotoxic activity of NY9-specific CTL clones against 721.221-CD4-HLA-B*3501 cells infected with the wild-type (NL-432) or mutant viruses. The frequencies of p24 antigen-positive cells among 721.221-CD4-HLA-B*3501 cells infected with NL-432, NL-432-Y181C, NL-432-Y181V, and NL-432-Y181I were 55%, 52%, 49%, and 48%, respectively. All data are shown as the means and ± SD of the results of triplicate assays. Statistical analysis was performed by using the *t* test. *, $P < 0.05$; **, $P < 0.01$ (as indicated by the brackets).

tant peptides more effectively than the wild-type one (Fig. 4B, right). The affinity of these mutant peptides for HLA-B*35:01 was higher than that of the NY9-3E peptides (Fig. 4C) (BL_{50} of 14.9 ± 0.7 μM for the NY9-3E WT versus 6.2 ± 1.3 μM for 3E7C [$P <$ 0.01], 1.4 ± 0.3 μM for 3E7V [$P < 0.01$], and 2.5 ± 0.4 μM for 3E7I [$P < 0.01$]), indicating that these mutations partially affected epitope recognition by TCRs on the T cells from KI-667 but not epitope recognition by those on the T cells from KI-805.

We further investigated the abilities of these HLA-B*35:01-rescricted CTLs to recognize target cells infected with the mutant viruses with the subtype A/E-derived mutations. The bulk T cells from KI-667 had a significantly lower ability to kill target cells infected with either mutant virus than to kill those infected with the WT virus (Fig. 4D, left). Since these T cells from KI-667 similarly recognized the target cells pulsed with the NY9-3E7I peptide and those pulsed with NY9-3E peptides (Fig. 4B, left), this 7I mutation may also have affected antigen presentation in cells infected with this mutant virus.

The bulk T cells from KI-805 showed different patterns of killing activity toward the target cells infected with these 3 mutant viruses: the killing activity for the target cells infected with the NL-432-$_{NY9\text{-}3E}$-Y181V, -Y181I, and -Y181C viruses was stronger than, similar to, and weaker than that seen with those infected with the wild-type virus, respectively (Fig. 4D, right). The T cells recognized the 7V and 7I mutant peptides more effectively than they did the wild-type peptide (Fig. 4B, right). The affinity of these mutant peptides was much higher than that seen with the wild-type peptide (Fig. 4C). These results suggest that enhanced affinity of these mutant peptides for HLA-B*35:01 resulted in effective recognition by the T cells of the cells infected with these viruses. The 7C mutation may have affected antigen presentation, since the 7C mutant peptide had higher affinity for HLA-B*35:01 than did the wild-type peptide (Fig. 4C) and the T cells similarly recognized both peptides (Fig. 4B, right). Thus, NY9-specific T cells showed differences in recognition of target cells infected with these mutant viruses not only between the subtype B-infected and subtype A/E-infected individuals but also among the subtype A/E-infected individuals.

Accumulation of resistance mutations at RT181 in individuals chronically infected with subtype A/E virus. To investigate whether RT181 drug resistance mutations had accumulated in antiretroviral-naive HIV-1-infected individuals, we analyzed 363

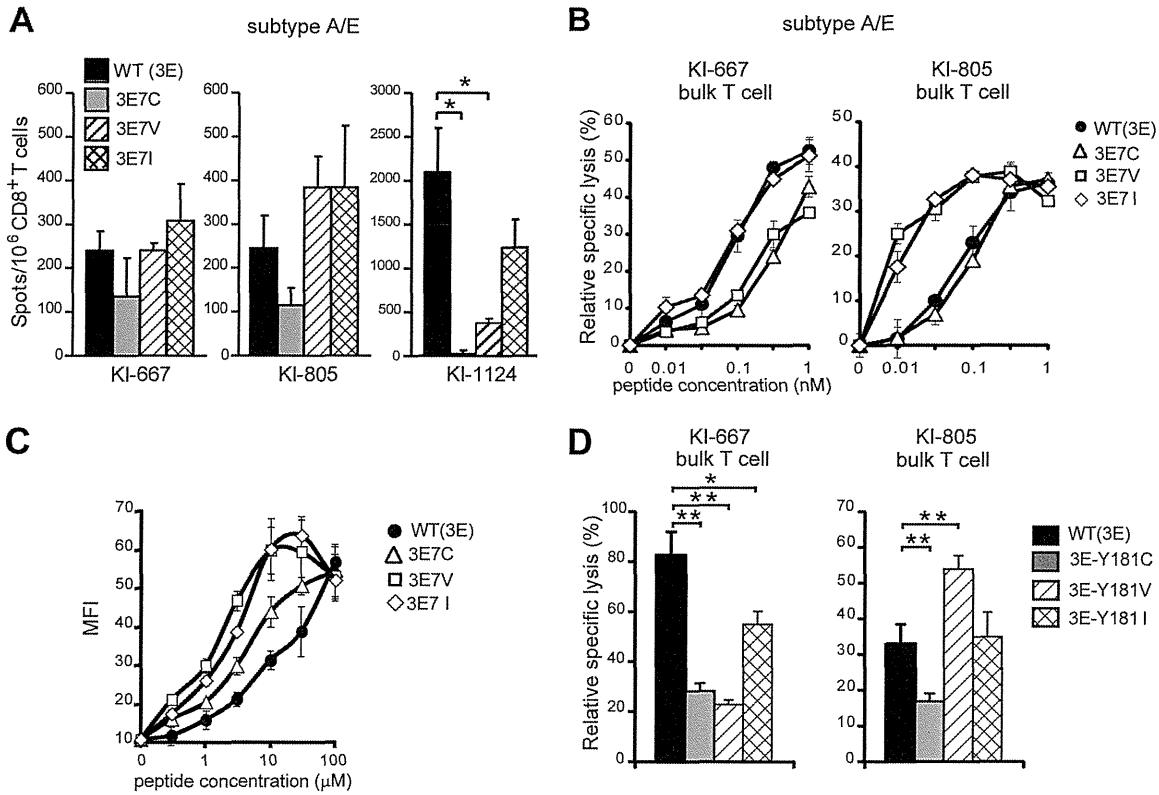

FIG 4 Effect of RT181 mutations on HLA-B*35:01-restricted NY9-3E-specific CTL recognition in subtype A/E infections. (A) *Ex vivo* $CD8^+$ T cell responses to NY9-3E (WT) or 3 mutant peptides in HLA-B*35:01$^+$ individuals infected with subtype A/E virus. $CD8^+$ T cell responses to NY9-3E or 3 mutant peptides in PBMCs from 3 HIV-1-infected individuals were analyzed by performing the ELISPOT assay using a 100 nM concentration of the peptides. (B) Relative cytotoxic activity of HLA-B*35:01-restricted NY9-3E-specific bulk T cells toward C1R-B*3501 cells prepulsed with WT or the mutant peptides. (C) Binding of the NY9-3E and mutant peptides to HLA-B*35:01. Binding ability was measured by performing the HLA class I stabilization assay using RMA-S-B*3501 cells. (D) Relative cytotoxic activity of NY9-3E-specific bulk T cells against 721.221-CD4-HLA-B*3501 cells infected with the wild-type (NL-432-$_{NY9\text{-}3E}$) or mutant viruses. The frequencies of p24 antigen-positive cells among 721.221-CD4-HLA-B*3501 cells infected with NL-432-$_{NY9\text{-}3E}$, NL-432-$_{NY9\text{-}3E}$-Y181C, NL-432-$_{NY9\text{-}3E}$-Y181V, and NL-432-$_{NY9\text{-}3E}$-Y181I were 46%, 49%, 52%, and 48%, respectively, for KI-667 and 60%, 58%, 59%, and 58%, respectively, for KI-805. All data in this figure are shown as the means and ± SD of the results of triplicate assays. Statistical analysis was performed by using the *t* test. *, $P < 0.05$; **, $P < 0.01$ (as indicated by the brackets).

treatment-naive Japanese individuals infected with the subtype B virus and 377 treatment-naive Vietnamese individuals infected with the subtype A/E virus. The RT181 mutations were not detected in the Japanese (32), whereas the Y181C mutation was observed in 0.7% and 2.6% of the Vietnamese individuals recruited in 2013 and 2014, respectively (Table 2), suggesting that this mutation might have been accumulating in the Vietnam cohort. Only one individual with this mutation had HLA-A*02:01, and this mutation was not associated with allele HLA-A*02:01, HLA-B*35:01, or HLA-C*12:02 (data not shown), indicating that the mutation was not selected by CTL restricted by these HLA alleles. Since this mutation reduced the recognition of 2 epitopes by T cells specific for these epitopes in the subtype A/E infection, the accumulation of this mutation may influence the control of HIV-1 by HIV-1-specific CTLs.

DISCUSSION

According to the Stanford HIV database, Y181C, Y181V, and Y181I mutations accumulated in 17%, 0.4%, and 0.9% of 10,958 NNRTI-treated subtype B virus-infected patients, respectively,

TABLE 2 Frequency of RT181 drug mutation in antiretroviral-naive Japanese and Vietnamese individuals

Mutation	No. (%) of individuals infected with:								
	Japan subtype B					Hanoi subtype A/E			
		Yr of sampling					Yr of sampling		
	Total (n = 363)	2008 (n = 68)	2009 (n = 121)	2010 (n = 129)	2011 (n = 45)	Total (n = 377)	2012 (n = 118)	2013 (n = 143)	2014 (n = 116)
Y181C	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (1.1)	0 (0)	1 (0.7)	3 (2.6)
Y181V	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Y181I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

while the frequencies of these mutations were 36%, 2.1%, and 2.0%, respectively, among 1,679 subtype A/E-infected patients (33). In Kenya, 15% (8/55) of subtype A-infected patients who had undergone virologic failure had the Y181C, Y181V, or Y181I mutation (34). In China, 11.8% of 515 CRF08_BC, CRF07_BC, and CRF01_AE patients with virologic failure had the Y181C mutation, and 0.6% of them had the Y181V mutation (35). Moreover, 32% of 48 Senegalese children with virologic failure had the Y181C mutation (36). Further large-scale analysis in Spain showed that the rates of prevalence of Y181C, Y181I, and Y181V were 21.8%, 0.5%, and 0.2%, respectively, in 1,064 HIV-1-infected patients failing EFV, NVP, or ETR therapy (37). The Y181C mutation, which requires a single nucleotide change, is frequently observed in NNRTI-treated patients compared to Y181I or Y181V, each of which requires a double-nucleotide change. Thus, the NNRTI-resistance Y181C/V/I mutations, especially Y181C, have been accumulating in NNRTI-treated HIV-1 subtype B-infected and subtype A/E-infected patients.

The Y181C mutation is known to decrease virus replication capacity (38, 39), whereas the Y181I mutation has no effect on replication capacity (39, 40). Although no one has reported on the replication kinetics of the Y181V mutation in the absence of drugs, this Y181V mutation confers a clear fitness advantage over other NNRTI-resistance mutations in the presence of ETR (41). Drug treatment often increases the risk of subsequent transmission of drug resistance in new cases of an HIV infection. The Y181C mutation was also reported in treatment-naive individuals among different cohorts (see Table S1 in the supplemental material) at 2.3% (1/43), 1.1% (1/91), 0.6% (1/156), 3.0% (3/101), and 1.6% (2/129) of treatment-naive subtype C-infected individuals in Mozambique (42), in South India (43), in KwaZulu-Natal Provinces in South Africa (44), in Tanzania (45), and in China (46), respectively, and 0.43% of 1,389 treatment-naive individuals in southern Vietnam, where subtype A/E virus was the most prevalent subtype (47). The Y181C mutation or the Y181C/I mutation was detected in 0.4%, 0.3% (1/303), 0.2% (4/2,655), and 1.8% (6/336) of treatment-naive individuals in northern Poland (48), Brazil (49), Mexico (50), and Hondurans (51), respectively, where subtype B was the most prevalent. In sub-Saharan African, including six countries where a prevalence of subtypes C, A, D, and A/G was found, 0.7% of 2,436 treatment-naive patients had Y181C/I mutations (52). The present study also demonstrated that 1.1% of 377 treatment-naive individuals in the Vietnam cohort recruited from 2012 to 2014 had the Y181C mutation. The finding that the frequency of this mutation increased to 2.6% in 2014 indicates that this mutation had been accumulating in Hanoi. Since most HIV-1-infected patients are treated with NVP and EFV in these countries, including Vietnam, the accumulation of this mutation in treatment-naive patients may be due to transmission from those who had received treatment with these drugs.

The analysis of *ex vivo* T cells by use of the ELISPOT assay showed that responses of HLA-B*35:01-restricted NY9-specific T cells to the 3 mutant peptides (NY9-7C, NY9-7V, and NY9-7I) was significantly weaker than that to the WT peptide in HIV-1 subtype B-infected HLA-B*35:01$^+$ individuals. Furthermore, a NY9-specific CTL clone from an HIV-1 subtype B-infected HLA-B*35:01$^+$ individual failed to recognize these 3 mutant peptides and target cells infected with these mutant viruses. These results indicate that these 3 mutations affected epitope recognition by NY9-specific T cells in HIV-1 subtype B infections. On the other hand, these mutations exhibited effects on the recognition by T cells specific for the subtype A/E-derived NY9-3E epitope that were different from the effects on the recognition by T cells specific for the subtype B-derived NY9 epitope. A previous study showed that the substitution from D to E at position 3 of the NY9 epitope critically reduces the binding of this epitope to HLA-B*35:01 molecules (25). In addition, the binding affinity of the subtype B-derived NY9 mutant peptides for HLA-B*35:01 was mostly similar to that of the WT peptide, whereas that of the subtype A/E-derived NY9-3E mutant peptides for HLA-B*35:01 was higher than that of the WT (NY9-3E) peptide. Taken together, these results suggest that the difference in HLA-peptide binding affinity may be involved in the different T cell recognition patterns for RT181 mutations between the subtype B and the subtype A/E infections.

In the present study, we demonstrated that the 3 NNRTI-resistance mutations affected the 3 different patterns of HIV-1 epitope-specific T cell recognition and furthermore exhibited different effects on epitope recognition by HLA-B*35:01-restricted NY9-specific T cells between the subtype B and the A/E infections. The Y181C mutation was predominantly detected in NNRTI-treatment patients and even in treatment-naive individuals. The Y181C mutation may influence the control of HIV-1 by the CTLs in Vietnam, since this mutation is accumulating in treatment-naive Vietnamese.

ACKNOWLEDGMENTS

This research was supported by the Global COE program "Global Education and Research Center Aiming at the Control of AIDS," launched as a project commissioned by the Ministry of Education, Culture, Sports, Science and Technology, Japan; by Grants-in-Aid for AIDS Research from the Ministry of Health, Labour, and Welfare and from the Ministry of Education, Culture, Sports, Science and Technology, Japan; and by a Joint Research Grant with the Institute of Tropical Medicine, Nagasaki University.

We thank Sachiko Sakai for her secretarial assistance.

We have no financial conflicts of interest.

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Tenofovir alafenamide versus tenofovir disoproxil fumarate, coformulated with elvitegravir, cobicistat, and emtricitabine, for initial treatment of HIV-1 infection: two randomised, double-blind, phase 3, non-inferiority trials

*Paul E Sax, David Wohl, Michael T Yin, Frank Post, Edwin DeJesus, Michael Saag, Anton Pozniak, Melanie Thompson, Daniel Podzamczer, Jean Michel Molina, Shinichi Oka, Ellen Koenig, Benoit Trottier, Jaime Andrade-Villanueva, Gordon Crofoot, Joseph M Custodio, Andrew Plummer, Lijie Zhong, Huyen Cao, Hal Martin, Christian Callebaut, Andrew K Cheng, Marshall W Fordyce, Scott McCallister, for the GS-US-292-0104/0111 Study Team**

Summary

Background Tenofovir disoproxil fumarate can cause renal and bone toxic effects related to high plasma tenofovir concentrations. Tenofovir alafenamide is a novel tenofovir prodrug with a 90% reduction in plasma tenofovir concentrations. Tenofovir alafenamide-containing regimens can have improved renal and bone safety compared with tenofovir disoproxil fumarate-containing regimens.

Methods In these two controlled, double-blind phase 3 studies, we recruited treatment-naive HIV-infected patients with an estimated creatinine clearance of 50 mL per min or higher from 178 outpatient centres in 16 countries. Patients were randomly assigned (1:1) to receive once-daily oral tablets containing 150 mg elvitegravir, 150 mg cobicistat, 200 mg emtricitabine, and 10 mg tenofovir alafenamide (E/C/F/tenofovir alafenamide) or 300 mg tenofovir disoproxil fumarate (E/C/F/tenofovir disoproxil fumarate) with matching placebo. Randomisation was done by a computer-generated allocation sequence (block size 4) and was stratified by HIV-1 RNA, CD4 count, and region (USA or ex-USA). Investigators, patients, study staff, and those assessing outcomes were masked to treatment group. All participants who received one dose of study drug were included in the primary intention-to-treat efficacy and safety analyses. The main outcomes were the proportion of patients with plasma HIV-1 RNA less than 50 copies per mL at week 48 as defined by the the US Food and Drug Adminstration (FDA) snapshot algorithm (pre-specified non-inferiority margin of 12%) and pre-specified renal and bone endpoints at 48 weeks. These studies are registered with ClinicalTrials.gov, numbers NCT01780506 and NCT01797445.

Findings We recruited patients from Jan 22, 2013, to Nov 4, 2013 (2175 screened and 1744 randomly assigned), and gave treatment to 1733 patients (866 given E/C/F/tenofovir alafenamide and 867 given E/C/F/tenofovir disoproxil fumarate). E/C/F/tenofovir alafenamide was non-inferior to E/C/F/tenofovir disoproxil fumarate, with 800 (92%) of 866 patients in the tenofovir alafenamide group and 784 (90%) of 867 patients in the tenofovir disoproxil fumarate group having plasma HIV-1 RNA less than 50 copies per mL (adjusted difference 2·0%, 95% CI −0·7 to 4·7). Patients given E/C/F/tenofovir alafenamide had significantly smaller mean serum creatinine increases than those given E/C/F/tenofovir disoproxil fumarate (0·08 *vs* 0·12 mg/dL; p<0·0001), significantly less proteinuria (median % change −3 *vs* 20; p<0·0001), and a significantly smaller decrease in bone mineral density at spine (mean % change −1·30 *vs* −2·86; p<0·0001) and hip (−0·66 *vs* −2·95; p<0·0001) at 48 weeks.

Interpretation Through 48 weeks, more than 90% of patients given E/C/F/tenofovir alafenamide or E/C/F/tenofovir disoproxil fumarate had virological success. Renal and bone effects were significantly reduced in patients given E/C/F/tenofovir alafenamide. Although these studies do not have the power to assess clinical safety events such as renal failure and fractures, our data suggest that E/C/F/tenofovir alafenamide will have a favourable long-term renal and bone safety profile.

Funding Gilead Sciences.

Published Online
April 16, 2015
http://dx.doi.org/10.1016/
S0140-6736(15)60616-X

See Online/Comment
http://dx.doi.org/10.1016/
S0140-6736(15)60725-5

*Study investigators are listed in the appendix.

Division of Infectious Diseases and Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA, USA (Prof P E Sax MD); Department of Medicine, University of North Carolina, Chapel Hill, NC, USA (D Wohl MD); Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, NY, USA (Prof M T Yin MD); Department of HIV Medicine, King's College, Hospital NHS Foundation Trust, London, UK (F Post MD); Orlando Immunology Center, Orlando, FL, USA (E DeJesus MD); Department of Medicine, University of Alabama Birmingham, Birmingham, AL, USA (M Saag MD); Department of Medicine, Chelsea and Westminster Hospital, NHS Foundation Trust, London, UK (A Pozniak MD); AIDS Research Consortium of Atlanta, Atlanta, GA, USA (M Thompson MD); HIV Unit, Infectious Disease Service. Hospital Universitari de Bellvitge, Barcelona, Spain (D Podzamczer MD); Hôpital Saint Louis, Paris, France (Prof J M Molina MD); AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan (S Oka MD); Instituto Dominicano de Estudios Virologicos (IDEV), Santo Domingo, Dominican

Introduction

Guidelines for initial treatment of HIV-1 infection recommend the use of two nucleoside reverse transcriptase inhibitors plus a third active drug from a different class.[1] Of nucleoside reverse transcriptase inhibitors, tenofovir disoproxil fumarate is included in most recommended regimens. Although potent and generally well tolerated, tenofovir disoproxil fumarate can cause clinically significant renal toxic effects,[2] especially in patients with risk factors for kidney disease or who are receiving concomitant ritonavir-boosted protease inhibitors.[3,4] Additionally, tenofovir disoproxil fumarate has been associated with greater reductions in bone mineral density than other antiretroviral drugs.[5] In one observational study,[6]

Republic (E Koenig MD); Clinique Medicale L'Actuale in Montreal, Montreal, Canada (B Trottier MD); Unidad de VIH del Hospital Civil de Guadalajara, CUCS, U de G Guadalajara, Mexico (J Andrade-Villanueva MD); Gordon Crofoot Research, Houston, TX, USA (G Crofoot MD); and Gilead Sciences, Foster City, CA, USA (J M Custodio PharmD, A Plummer MS, L Zhong PhD, H Cao MD, H Martin MD, C Callebaut PhD, A K Cheng MD, M W Fordyce MD, S McCallister MD)

Correspondence to: Dr Paul E Sax, Division of Infectious Diseases and Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA 02115, USA
psax@partners.org

Research in context

Evidence before this study

Although potent and generally well tolerated, tenofovir disoproxil fumarate might lead to clinically significant renal and bone disease. The risk of these side-effects is related to plasma concentrations of tenofovir. The novel tenofovir prodrug tenofovir alafenamide delivers 90% lower plasma tenofovir compared with standard tenofovir disoproxil fumarate. This pharmacology might reduce the off-target effects of tenofovir, in particular renal and bone toxicity. A phase 2 comparative trial of tenofovir alafenamide versus tenofovir disoproxil fumarate (both coformulated with elvitegravir, cobicistat, and emtricitabine [E/C/F]) showed similar efficacy of tenofovir alafenamide and tenofovir disoproxil fumarate with a significantly reduced effect on estimated glomerular filtration rate, tubular proteinuria, and bone mineral density. We did a systematic search of PubMed to explore the use of tenofovir alafenamide in treatment-naive patients, with a particular focus on renal and bone safety in treatment-naive patients. Search terms included "tenofovir alafenamide" AND "naive" AND "renal" OR "bone." Searches were limited to articles published in English between 1997 and March, 2015. Only one article was retrieved, which was the phase 2 randomised clinical trial comparing E/C/F/tenofovir alafenamide with E/C/F/tenofovir disoproxil fumarate.

Added value of this study

These two fully-powered phase 3 double-blind, international clinical trials compared single-tablet regimens of E/C/F/tenofovir alafenamide with E/C/F/tenofovir disoproxil fumarate, with results confirming the earlier findings. Both regimens showed higher than 90% efficacy, with low (<1%) rates of discontinuations due to adverse events. Compared with tenofovir disoproxil fumarate, tenofovir alafenamide treatment led to smaller decreases in estimated glomerular filtration rate, less proteinuria (significant for all types measured), and had a more favourable effect on hip and spine bone mineral density. All lipid fractions increased more in the tenofovir alafenamide than in the tenofovir disoproxil fumarate group with similar total to HDL cholesterol ratios.

Implications of all the available evidence

E/C/F/tenofovir alafenamide is a highly effective regimen for treatment-naive patients, with more favourable effects than E/C/F/tenofovir disoproxil fumarate on renal and bone health. The hope is that these findings will translate into improved safety of tenofovir alafenamide-based antiretroviral therapy over years of treatment while maintaining a similarly high efficacy rate.

investigators noted that tenofovir disoproxil fumarate exposure was associated with an increased rate of fractures.

As a prodrug, tenofovir disoproxil fumarate is initially metabolised to tenofovir, which is subsequently metabolised in cells to tenofovir-diphosphate. Although intracellular tenofovir-diphosphate is responsible for the drug's antiviral activity, higher circulating plasma levels of tenofovir have been associated with an increased risk of both renal and bone toxicity.[7–10] A novel tenofovir prodrug tenofovir alafenamide results in roughly four times higher intracellular concentrations of the active metabolite tenofovir-diphosphate compared with tenofovir disoproxil fumarate, allowing for much lower doses of tenofovir alafenamide versus tenofovir disoproxil fumarate.[11] Because of tenofovir alafenamide's reduced dose and the improved stability, plasma exposure of tenofovir is 90% lower with tenofovir alafenamide than with tenofovir disoproxil fumarate, which is believed to reduce the risk of renal and bone toxicity.[7]

Findings of a phase 2 comparative trial[12] of tenofovir alafenamide versus tenofovir disoproxil fumarate (both coformulated with elvitegravir, cobicistat, and emtricitabine) showed similar antiviral activity of tenofovir alafenamide and tenofovir disoproxil fumarate, with a significantly reduced effect of tenofovir alafenamide compared to tenofovir disoproxil fumarate on estimated glomerular filtration rate, tubular proteinuria, and bone mineral density. To confirm these findings, we did two phase 3, double-blind clinical trials comparing elvitegravir, cobicistat, emtricitabine, and tenofovir alafenamide (E/C/F/tenofovir alafenamide) with elvitegravir, cobicistat, emtricitabine, and tenofovir disoproxil fumarate (E/C/F/tenofovir disoproxil fumarate), with a protocol-specified focus on renal and bone safety.

Methods

Study design and patients

GS-US-292-0104 and GS-US-292-0111 are randomised, double-blind, multicentre, active-controlled phase 3 trials done at 134 sites in North America, Europe, Australia, Japan, and Thailand (GS-US-292-0104), and 128 sites in North America, Europe, and Latin America (GS-US-292-0111). Studies were undertaken in accordance with the Declaration of Helsinki and were approved by central or site-specific review boards or ethics committees. All patients gave written informed consent. Adults (aged ≥18 years) were enrolled if they had HIV-1 and no previous antiretroviral treatment, had HIV-1 RNA concentration of at least 1000 copies per mL, and an estimated glomerular filtration (creatinine clearance, Cockcroft-Gault) rate of at least 50 mL per min. Eligible patients had a screening HIV-1 genotype showing sensitivity to elvitegravir, emtricitabine, and tenofovir. No CD4 entry criteria were used. We excluded patients with positive hepatitis B surface antigen or hepatitis C antibody or a new AIDS-defining illness within 30 days of screening.

Randomisation and masking

Eligible patients were randomly assigned (1:1) to receive either coformulated 150 mg elvitegravir, 150 mg cobicistat,

www.thelancet.com Published online April 16, 2015 http://dx.doi.org/10.1016/S0140-6736(15)60616-X

200 mg emtricitabine, and 10 mg tenofovir alafenamide once a day, or coformulated 150 mg elvitegravir, 150 mg cobicistat, 200 mg emtricitabine, and 300 mg tenofovir disoproxil fumarate once a day. Both regimens were given with food. Patients also received placebo tablets matching the alternative treatment; thus, investigators, patients, and study staff giving treatment, assessing outcomes, and collecting data were masked to treatment group. A computer-generated allocation sequence (block size 4) was created by Bracket (San Francisco, CA, USA), and randomisation was stratified by HIV-1 RNA (≤100 000 copies per mL, >100 000 to ≤400 000 copies per mL, or >400 000 copies per mL), CD4 count (<50 cells per μL, 50–199 cells per μL, or ≥200 cells per μL), and region (USA or ex-USA) at screening. Study investigators determined eligibility, obtained a participant number, and received automated treatment assignment based on a randomisation sequence.

Procedures

Post-baseline study visits occurred at weeks 2, 4, 8, 12, 16, 24, 36, and 48, after which patients continued masked treatment with visits every 12 weeks until week 96. After the primary endpoint had been reached, masked treatment with study drug was extended to week 144. Laboratory tests included haematological analysis, serum chemistry tests, fasting lipid parameters, CD4 counts, measures of renal function (estimated glomerular filtration rate, urine protein to creatinine ratio, urine albumin to creatinine ratio, retinol binding protein to creatinine ratio, β2-microglobulin to creatinine ratio, fractional excretion of uric acid, and fractional excretion of phosphate; Covance Laboratories, Indianapolis, IN, USA), and measurement of HIV RNA concentration (Roche TaqMan 2.0; Roche Diagnostics, Rotkreuz, Switzerland).

We used definitions of suboptimum virological response (<1 $\log_{10}$ reduction from baseline HIV-1 RNA and ≥50 copies per mL at the week 8 visit, confirmed at a subsequent visit) and virological rebound (plasma HIV-1 RNA <50 copies per mL, then having HIV-1 RNA ≥50 copies per mL, confirmed at a subsequent visit) to assess virological response. We defined virological failure as plasma HIV-1 RNA greater than or equal to 50 copies per mL and less than 1 $\log_{10}$ reduction from baseline at week 8, or 50 copies per mL or more HIV-1 RNA after previous suppression to less than 50 copies per mL or more than a 1 $\log_{10}$ increase in HIV-1 RNA from nadir. Any participant meeting these criteria had a second, confirmatory sample drawn within 3–6 weeks. Confirmatory samples with 400 copies per mL or more HIV-1 RNA were sent for HIV-1 genotype and phenotype analysis (PhenoSenseGT for Protease and Reverse Transcriptase genes, GenSeq Integrase and Phenosense Integrase for the Integrase gene; Monogram Biosciences, South San Francisco, CA, USA).

In all patients, dual energy x-ray absorptiometry scans of the lumbar spine and hip were done at baseline, week 24, and week 48 to measure percent changes in bone mineral density. The scans were processed by BioClinica (Newton, PA, USA). The preliminary results were reviewed twice by an independent data monitoring committee when half of patients had completed week 12 and when all patients had completed week 24 of follow-up, respectively. The primary endpoint analysis was done after all enrolled patients had completed their week 48 study visit or had prematurely discontinued study drug.

The primary endpoint was the proportion of patients who had plasma HIV-1 RNA less than 50 copies per mL at week 48 as defined by the the US Food and Drug Adminstration (FDA) snapshot algorithm.[13] Four key safety endpoints were pre-specified with multiplicity adjustments: hip bone mineral density, spine bone mineral density, serum creatinine, and treatment-emergent proteinuria. Additional secondary endpoints included treatment responses by subgroups, proportion of patients with plasma HIV-1 RNA less than 50 copies per mL when classifying missing as failure and missing as excluded, patients with HIV-1 RNA less than 20 copies per mL by snapshot, and change in CD4 count from baseline.

Statistical analysis

These two phase 3 studies were combined for a pre-specified pooled efficacy and safety analysis. Within each phase 3 study, for each of two interim analyses done for the independent data monitoring committee meeting, an α of 0·00001 was spent. Therefore, the significance level for the 1-sided non-inferiority test in the primary analysis at week 48 was 0·02499, equivalent to a two-sided 95·002% CI. The percentage differences and the associated 95·002% CIs were computed with the baseline HIV-1 RNA concentration and region stratum adjusted Mantel-Haenszel proportions.[14] To control for the overall type I error in the assessment of the primary efficacy endpoint and the four key safety endpoints, hypothesis testing was done in sequential order. The primary hypothesis of non-inferiority of E/C/F/tenofovir alafenamide relative to E/C/F/tenofovir disoproxil fumarate, with respect to the proportion of patients with less than 50 copies per mL of HIV-1 RNA at week 48 (as defined by the FDA snapshot algorithm) was tested first. The non-inferiority test was done at a one-sided, 0·02499 α level. If noninferiority was established, multiplicity adjustments were undertaken for the following safety endpoints with a fallback procedure[15] in the sequential order given below with prespecified two-sided α levels: hip bone mineral density (α=0·02), spine bone mineral density (α=0·01), serum creatinine (α=0·01998), and treatment-emergent proteinuria (α=0·00). The adjusted α levels were dependent on the results from preceding tests. For all the four safety endpoints, two-sided superiority tests were done.

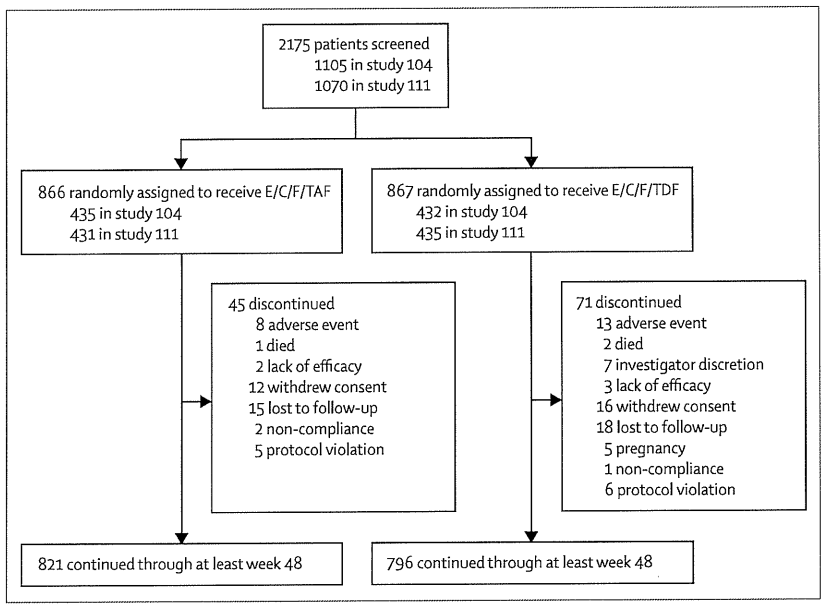

Figure 1: **Trial profile**
E/C/F/TAF=elvitegravir, cobicistat, emtricitabine, tenofovir alafenamide. E/C/F/TDF=elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate.

	Elvitegravir, cobicistat, emtricitabine, tenofovir alafenamide (n=866)	Elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate (n=867)
Age (years)	33 (26–42)	35 (28–44)
Women	133 (15%)	127 (15%)
Ethnic origin		
White	485 (56%)	498 (57%)
Black or African heritage	223 (26%)	213 (25%)
Hispanic or Latino	167 (19%)	167 (19%)
Asian	91 (11%)	89 (10%)
HIV disease status		
Asymptomatic	780 (90%)	802 (93%)
Symptomatic	53 (6%)	35 (4%)
AIDS	30 (4%)	26 (3%)
HIV risk factor		
Heterosexual sex	210 (24%)	219 (25%)
Homosexual sex§	652 (75%)	645 (74%)
Intravenous drug use	5 (1%)	6 (1%)
Median HIV-1 RNA (log_{10} c/mL)	4·58 (4·04–4·95)	4·58 (4·15–4·96)
HIV-1 RNA concentration >100 000 copies per mL	196 (23%)	195 (22%)
Median CD4 count (cells per μL)	404 (283–550)	406 (291–542)
Number with CD4 cell count (cells per μL)		
<50	24 (3%)	27 (3%)
≥50 to <200	88 (10%)	90 (10%)
≥200	753 (87%)	750 (87%)
Median estimated glomerular filtration rate (Cockcroft-Gault; mL/min)	117 (100–136)	114 (99–134)
Median BMI (kg/m²)	24·4 (22·0–28·0)	24·5 (21·7–28·0)

Data are median (IQR) or n (%).

Table 1: **Baseline characteristics**

For pooled data, assessment of non-inferiority of E/C/F/tenofovir alafenamide compared with E/C/F/tenofovir disoproxil fumarate was done with a two-sided 95% CI (α level not adjusted), with a prespecified non-inferiority margin of 12%. In the snapshot analysis using full analysis set that included all participants randomly assigned and receiving at least one dose of study drug, participants with less than 50 copies per mL of HIV-1 RNA between days 294 and 377 (week 48 window) were classified as successes. Participants with missing HIV-1 RNA data for the week 48 analysis window, who discontinued study drug, or who changed treatment before week 48 were classified as failures. A sample size of 840 patients in each study provided at least 95% power to establish non-inferiority between the two treatment groups with an overall response rate of 85% for viral suppression at week 48. Sample sizes were calculated with nQuery Advisor (version 6.0).

We did a prespecified, per-protocol snapshot analysis, which included all participants who enrolled, received at least one dose of study drug, and did not meet any of the following prespecified criteria: discontinuation of study drug before week 48 or HIV RNA data missing in week 48 analysis window, and adherence in the bottom 2·5th percentile.

Change from baseline in CD4 cell count at week 48 was summarised by treatment group with descriptive statistics based on recorded, on-treatment data in the full analysis set. The differences in changes from baseline in CD4 cell count between treatment groups and the 95% CI were constructed with analysis of variance model, including baseline HIV-1 RNA and region as fixed covariates in the model.

The safety population included all randomly assigned patients who received at least one dose of study drug. All safety data are described in summary form on all data collected after the date study drug was first given and up to 30 days after the last dose of study drug, if the participant discontinued treatment. Adherence to the investigational antiretroviral regimens was computed as number of pills taken divided by number of pills prescribed. Adverse events were coded with the Medical Dictionary for Regulatory Activities (version 17.0). We used Fisher's exact test to compare treatment differences for adverse events and Wilcoxon rank sum test to compare treatment differences for continuous laboratory test results (SAS; version 9.2).

These studies were done according to protocol without significant deviations and are registered with ClinicalTrials.gov, numbers NCT01780506 and NCT01797445.

Outcomes

The main outcomes were the proportion of patients with plasma HIV-1 RNA less than 50 copies per mL (non-inferiority margin of 12%) and pre-specified renal and bone endpoints at 48 weeks (centrally assessed). Secondary outcomes were percentage change from