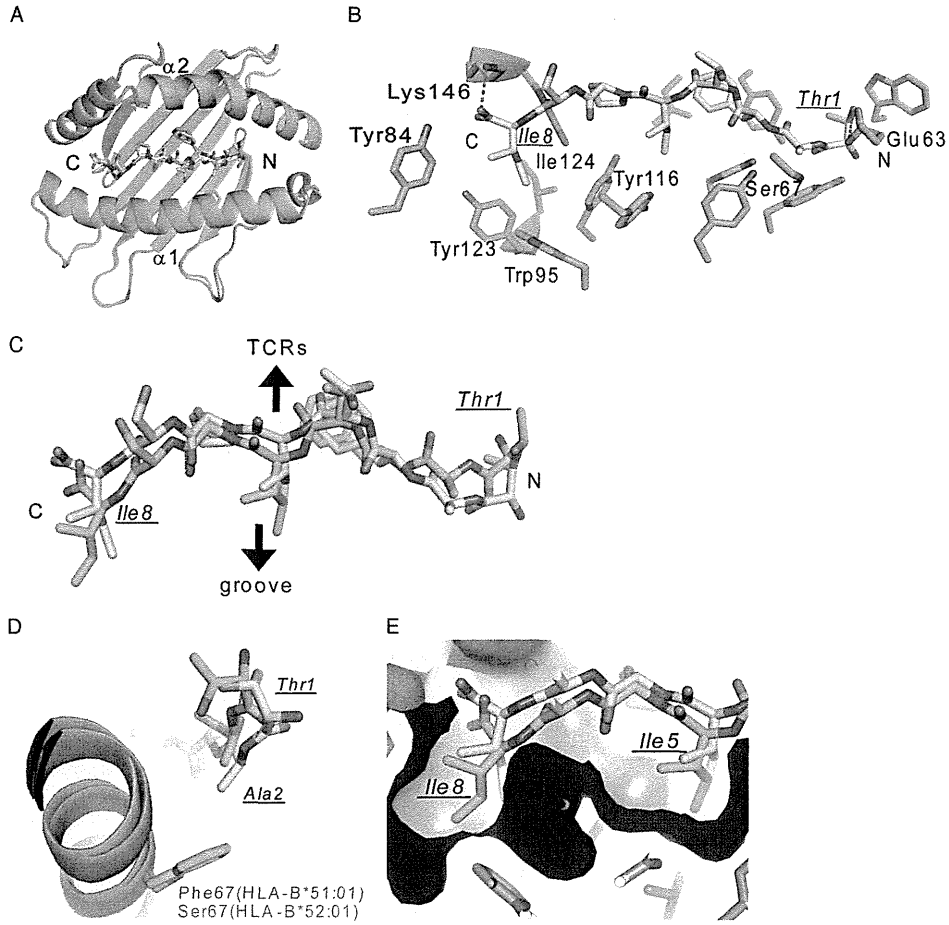

FIG 6 Structural comparison of HLA-B*52:01 and HLA-B*51:01 molecules complexed with the Pol283-8 peptide. (A) Crystal structures of HLA α1-α2 domains complexed with the Pol283-8 peptide (stick model) on the HLA-B*52:01 (green, yellow) and HLA-B*51:01 (cyan, cyan) complexes. This same coloring also applies to panels B to E. (B) Pol283-8 peptide and interacting side chains on the HLA-B*52:01 complex. Hydrogen bonds are shown as blue dotted lines. (C) Comparison of the Pol283-8 peptide conformations of HLA-B*52:01 and HLA-B*51:01 complexes. (D) N-terminal side of HLA-B*52:01 and HLA-B*51:01 complexes. (E) C-terminal side of HLA-B*52:01 and HLA-B*51:01 complexes. Surface presentation for the α1-α2 domains is shown in gray.

we had previously reported (45). This finding explains the cross presentation of this peptide by both HLA alleles. On the other hand, there was a notable conformational difference in the N-terminal region of the peptide between the two alleles (Fig. 6C and D). The replacement of Phe67 of HLA-B*51:01 with Ser in HLA-B*52:01 makes a local space, causing the N-terminal region of the peptide (T1 and A2) to reside deeper in the peptide-binding groove. Furthermore, the Gln63Glu mutation in HLA-B*52:01 affords a new interaction with the T1 residue of the peptide. These changes would, to some extent, have hidden the side chains of T1 and A2 (flat surface) from the TCRs, which may have reduced their interactions with TCRs on the HLA-B*52:01-restricted CTLs. On the other hand, the conformation of the C-terminal region of the peptide complexed with HLA-B*51:01 or HLA-B*52:01 was similar, even though C-terminal Ile8 of the peptide exhibited shallower penetration of the hydrophobic groove in the case of HLA-B*52:01 than in that of HLA-B*51:01 (Fig. 6C and E). These results may indicate that the relatively flat surface of the N-terminal side of the peptide contributed to the lower affinity for TCRs in the case of HLA-B*52:01.

DISCUSSION

HLA-B*52:01 and HLA-B*51:01 differ by only two residues, at positions 63 and 67 (44). Substitutions at these residues affect the formation of the B pocket in the peptide-binding pocket (45), suggesting the possibility that HLA-B*52:01 has a peptide motif different from that of HLA-B*51:01. Indeed, HLA-B*52:01-binding peptides have P2 primary anchors that are different from HLA-B*51:01-binding ones (30, 46). Since the Pol283-8 epitope carries Ala at its second position and Ile at the C terminus of the peptide, it is likely that this peptide would effectively bind to HLA-B*51:01 but not to HLA-B*52:01. However, the results of the HLA stabilization assay demonstrated that the Pol283-8 peptide did effectively bind to HLA-B*52:01. Since the HLA-B*52:01-binding peptide is known to have Pro as its preferred P2 anchor residue, this peptide carrying Ala at position 2 may be capable of binding to HLA-B*52:01. A previous study showed cross-recognition of alloreactive T cells between HLA-B*51:01 and HLA-B*52:01 (47, 48), indicating that some self-peptides can be presented by both of these HLA class I molecules. The findings on the crystal structure

TABLE 3 Numbers and frequencies of individuals having I135X mutations in a Japanese cohort and a predominantly Caucasian cohort

Cohort	No./total no. (%) of individuals				
	B*51:01$^+$ B*52:01$^-$	B*51:01$^-$ B*52:01$^+$	B*51:01$^+$ B*52:01$^+$	B*51:01$^-$ B*52:01$^-$	Total
Japanese	51/51 (100)	42/49 (85.7)	5/5 (100)	88/151 (58.3)	186/256 (72.6)
Caucasian	125/131 (95.4)	17/26 (65.4)	0/0	331/1,198 (27.6)	473/1,355 (34.9)

of the HLA-B*52:01 molecule complexed with the Pol283-8 peptide clarified that HLA-B*52:01 could bind to the peptide in a fashion similar to but slightly different from that of HLA-B*51:01. These findings support the presentation of the Pol283-8 peptide by both HLA-B*52:01 and HLA-B*51:01.

Pol283-8-specific CD8$^+$ T cells were detected in 7 of 14 HLA-B*52:01$^+$ HLA-B*51:01$^-$ individuals chronically infected with HIV-1. A previous analysis showed that CD8$^+$ T cells specific for this epitope are frequently detected in HLA-B*51:01$^+$ individuals chronically infected with HIV-1 (49). These results, taken together, indicate that this epitope is immunodominant in both HLA-B*51:01$^+$ and HLA-B*52:01$^+$ individuals. The analysis of 257 Japanese individuals revealed an association between HLA-B*52:01 and a variety of nonconsensus residues at RT codon 135 (I135X). Specifically, variants 8T, 8L, 8R, and 8V predominated in HLA-B*52:01$^+$ individuals, suggesting that these mutations had been selected by HLA-B*52:01-restricted CTLs. The viral suppression assay revealed that the HLA-B*52:01-restricted CTLs failed to suppress the replication of these mutant viruses. These results support the idea that the I135X mutation can be selected by immune pressure via Pol283-8-specific CTLs in HLA-B*52:01$^+$ individuals. Our previous studies showed that the 8L, 8T, and 8R mutations affected the recognition by Pol283-8-specific, HLA-B*51:01-restricted CTL clones (15, 28). These studies, together with the present study, indicate that accumulation of 8L, 8T, and 8R mutations in the HIV-infected Japanese population may be due to immune pressure by both HLA-B*52:01-restricted and HLA-B*51:01-restricted CTLs. Our analysis of the crystal structure of the HLA-B*52:01-peptide complex demonstrated that position 8 of the Pol283-8 peptide was deeply packed into the hydrophobic groove. Whereas the 8L, 8T, and 8R substitutions likely had a relatively large effect on the structure of the complex, the 8V mutation, resulting in only the deletion of the small methyl group, caused only very limited changes. Thus, the structural analysis supports the idea that the 8L, 8T, and 8R mutations affected the TCR recognition of the peptide and/or its binding to HLA-B*52:01.

The present study confirmed previous studies of nine worldwide cohorts (15) and a Chinese cohort (50) that showed a strong association of I135X with HLA-B*51:01. The I135X mutation was found in 58.3 and 27.6% of HLA-B*51:01$^-$ HLA-B*52:01$^-$ Japanese and predominantly Caucasian individuals, respectively (Table 3), supporting greater population level accumulation of this mutation in Japanese than in other cohorts. Since the Japanese cohort included twice as many HLA-B*51:01$^+$ individuals as the IHAC cohort (21.9% of Japanese and 9.4% of Caucasians in IHAC), the difference in the I135X variant frequency between these two cohorts would be driven, to a large extent, by the higher HLA-B*51:01 prevalence in the former than in the latter. The association of HLA-B*52:01 with this mutation was much weaker than that of HLA-B*51:01 in both cohorts but still highly statistically significant (an lnOR of 11.7 [$P = 8.77 \times 10^{-4}$] versus an lnOR of 40.0 [$P = 5.78 \times 10^{-12}$] in the Japanese cohort and an lnOR of 3.06 [$P = 2.95 \times 10^{-5}$] versus an lnOR of 5.71 [$P = 1.58 \times 10^{-51}$] in IHAC). Because of the relatively low B*52:01$^+$ frequency (~2%) in IHAC, the effect of HLA-B*52:01 on the overall prevalence of I135X was relatively low in this cohort. In contrast, in the Japanese cohort, where the HLA-B*52:01$^+$ prevalence was relatively high (>20%), this allele represents a major driving force behind 135X selection in this cohort. Thus, selection pressure from both HLA-B*51:01 and HLA-B*52:01 likely contributed to the observed population level accumulation of I135X mutations in the Japanese population.

Previous studies showed that HLA-B*51:01-restricted, Pol283-8-specific CTLs have a strong ability to suppress HIV-1 replication *in vitro* (28) and that they suppressed the replication of the 8V mutant virus but failed to suppress that of the 8T, 8L, and 8R mutant viruses (15). The frequency of the Pol283-8-specific CTLs was inversely correlated with the plasma viral load in HLA-B*51:01$^+$ hemophiliacs infected with HIV-1 approximately 30 years ago (28). The 8T, 8L, and 8R mutations did not affect replication capacity, whereas the 8V mutation conferred a modest fitness cost (15). These findings support the suppression of the wild-type or 8V mutant virus by Pol283-8-specific CTLs as a major mechanism of slow progression to AIDS in Japanese hemophiliacs. This CTL response was also elicited in Chinese HLA-B*51:01$^+$ individuals infected with the 8V mutant virus; furthermore, a low viral load and a high CD4 count were significantly associated with the presence of at least one of three HLA-B*51:01-restricted CTL responses, including a Pol283-8-specific one (50). Thus, these findings support the idea that Pol283-8-specific CTLs play an important role in the control of HIV-1 infection.

The present study demonstrated that HLA-B*52:01-restricted, Pol283-8-specific CTLs also had a strong ability to suppress HIV-1 replication *in vitro* (80% suppression at an E/T cell ratio of 0.3:1). However, the ability of HLA-B*52:01-restricted CTLs to suppress the replication of HIV-1 was weaker than that of HLA-B*51:01-restricted CTLs (Fig. 5B). Inspection of the crystal structures of both HLA molecules complexed with the Pol283-8 peptide suggests that the relatively shallow penetration of the hydrophobic groove of HLA-B*52:01 by the C-terminal side of the peptide, in contrast to the tightly packed binding with HLA-B*51:01, may have resulted in an unstable conformation of the complex. Furthermore, Ser67 of HLA-B*52:01 would have provided more space and loose interactions with the peptide than in the case of the Phe of HLA-B*51:01. Interestingly, the Pol283-8 peptide would have displayed only side chains of Thr1 and Ser7, and some part of the main chains, to CTLs. Therefore, these results suggest that the unstable backbone conformation and side chain positions in the case of HLA-B*52:01 largely contributed to the lower TCR affinity than that afforded by HLA-B*51:01. These results support that selection pressure *in vivo* via the HLA-B*52:01-restricted CTLs would be weaker than that via the HLA-B*51:01-restricted CTLs. Indeed, the prevalence of I135X mutations in HLA-B*51:

01^+ individuals was higher than that in HLA-B*52:01^+ individuals. The difference in the pattern of escape mutant selection by these CTLs between the HLA-B*51:01^+ and HLA-B*52:01^+ individuals might also have been due to the difference in their abilities to suppress HIV-1 replication. However, it still remains unclear why the 8T mutant was predominantly selected in the HLA-B*51:01^+ but not in the HLA-B*52:01^+ individuals. Further studies are expected to clarify the mechanism to explain how these CTLs selected different patterns of mutations at RT135.

Previous studies showed that the T242N mutant was selected by HLA-B*58:01-restricted and HLA-B*57-restricted CTLs specific for TW10 epitope in HIV-1 clade B-infected and clade C-infected individuals (25–27). Herein we also showed that I135X was selected by Pol283-8-specific CTLs restricted by two different HLA class I molecules. However, the strength and the pattern of the selection of I135X was different between HLA-B*51:01 and HLA-B*52:01. The present study suggests that this difference in the selection pattern was associated with that between the HLA-B*51:01^+ and HLA-B*52:01^+ individuals in terms of the ability of Pol283-specific CTLs to suppress HIV-1 replication. Thus, we characterized and experimentally validated distinct HIV-1 escape patterns of CTLs with the same epitope specificity and provided evidence that the extremely high prevalence of 135X in circulating Japanese sequences is likely driven not by one but by two HLA-B alleles.

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Minor contribution of HLA class I-associated selective pressure to the variability of HIV-1 accessory protein Vpu

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Viral evolution

ABSTRACT

Host HLA class I (HLA-I) allele-associated immune responses are major forces driving the evolution of HIV-1 proteins such as Gag and Nef. The viral protein U (Vpu) is an HIV-1 accessory protein responsible for CD4 degradation and enhancement of virion release by antagonizing tetherin/CD317. Although Vpu represents one of the most variable proteins in the HIV-1 proteome, it is still not clear to what extent HLA-I influence its evolution. To examine this issue, we enrolled 240 HLA-I-typed, treatment naïve, chronically HIV-infected subjects in Japan, and analyzed plasma HIV RNA nucleotide sequences of the vpu region. Using a phylogenetically-informed method incorporating corrections for HIV codon covariation and linkage disequilibrium among HLA alleles, we investigated HLA-associated amino acid mutations in the Vpu protein as well as in the translational products encoded by alternative reading frames. Despite substantial amino acid variability in Vpu, we identified only 4 HLA-associations in all possible translational products encoded in this region, suggesting that HLA-associated immune responses had minor effects on Vpu variability in this cohort. Rather, despite its size (81 amino acids), Vpu showed 103 codon–codon covariation associations, suggesting that Vpu conformation and function are preserved through many possible combinations of primary and secondary polymorphisms. Taken together, our study suggests that Vpu has been comparably less influenced by HLA-I-associated immune-driven evolution at the population level compared to other highly variable HIV-1 accessory proteins.

1. Introduction

Immune-mediated adaptation occurs during an HIV-1 infection. The HLA class I (HLA-I)-restricted $CD8^+$ cytotoxic T lymphocyte (CTL) response is one of the major forces driving HIV evolution, resulting in the selection of CTL escape mutants [1,2]. Despite the extensive genetic diversity of both HIV-1 and HLA-I alleles, escape pathways are reproducible and broadly predictable based on host HLA-I alleles [3–6]. Moreover, analysis of linked HLA-I and HIV datasets from large cohorts of HIV-infected subjects has facilitated our ability to map the landscape of immune escape mutations across HIV-1, identify immunogenic regions, and identify novel CTL epitopes [3,7].

Viral protein U (Vpu) is an accessory protein that is unique to HIV-1 and a subset of related simian immunodeficiency viruses. The HIV-1 Vpu protein has two major functions: degradation of newly synthesized CD4 molecules in the endoplasmic reticulum and enhancement of the release of progeny virions from infected cells by antagonizing tetherin/CD317, a host restriction factor that directly binds and retains viral particles on the surface of infected cells (reviewed in [8,9]). As such, Vpu is thought to play a role in virus spread and pathogenesis *in vivo*. Interestingly, Vpu is the most variable protein among all HIV proteins as evidenced by a cross-sectional comparison of HIV-1 sequences isolated from HIV-infected individuals [10], raising the possibility that Vpu undergoes adaptation in response to host immune responses. However, Vpu has been shown to be a minor target for CTLs as revealed by IFN-γ Elispot assays with overlapping peptides based on the subtype B consensus sequence [11]. Considering the highly variable nature of Vpu, it is possible to miss responses if the autologous virus sequence is markedly different from the peptide sequence when using this Elispot assay system [12].

In the present study, we sought to identify HLA-associated polymorphisms in Vpu and alternate reading frames and examine to

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what extent they are involved in Vpu amino acid variability at the population level. We utilize a published phylogenetic dependency network model [13], a comprehensive evolutionary model that considers all important confounding effects such as HIV phylogeny, HIV codon covariation, and linkage disequilibrium of HLA alleles.

2. Materials and methods

2.1. Patient samples

A total of 240 chronically HIV-1-infected, treatment-naïve subjects (CD4, median 237; IQR, 160-397; viral load, median 33,200; IQR, 222,000-55,400) followed at the AIDS Clinical Center, International Medical Center of Japan were enrolled in this study. All participants provided written informed consent. HLA-I typing was performed as previously described [14]. The most frequently observed *HLA-A, B,* and *C* alleles in this cohort were *HLA-A*24:02*, *HLA-B*52:01*, and *HLA-C*01:02*, respectively, consistent with HLA class I allelic frequencies of the Japanese people [14].

2.2. Sequence analysis of vpu

HIV-1 particles were precipitated by ultracentrifugation (50,000 rpm, 15 min) of patients' plasma, after which the viral RNA was extracted using standard methods. Following reverse transcription, DNA fragments encoding Vpu proteins were amplified by nested PCR, and gel purified as previously described [15,16]. The primers used were as follows: the primers for the first round of amplification were VVVa-F (5′-TTAAAAGAAAAGGGGG GATTGGGGG-3′) and VVVb-R (5′-ATTCCATGTGTACATT GTACTGT-3′); and those for the second round, VVVc-F (5′-AGATAATAGTGAC ATAAAAGTAGTGCCAAGAAG-3′) and VVVd-R (5′-CCATAATAGACT GTGACCCACAA-3′). The *vpu* sequence was then directly analyzed with an automated sequencer (Applied Biosystems 3500xL) and aligned to the *vpu* sequence of the HIV-1 subtype B reference strain HXB2 (Accession No. K03455). More than 90% of the subjects were infected with subtype B, as determined by phylogenetic analysis of concatenated sequences of *vif*, *vpr*, and *vpu* reading frames.

2.3. Analysis of amino acid sequence variability

A Shannon entropy score for each position in the Vpu protein was calculated and used to analyze amino-acid sequence variability, as described previously [10]. Entropy is a measure of the amino acid variability at a given position that takes into account both the number of possible amino acid residues allowed and their frequency.

2.4. Analysis of association between Vpu sequence polymorphisms and host HLA class I alleles

To identify HIV-HLA polymorphism associations, we employed a phylogenetically dependency network model [13], which comprehensively includes all confounding effects of the analysis, such as HIV founder effects, HIV codon co-variation, and linkage disequilibrium of HLA-I alleles. Multiple comparisons are addressed using q-values (refer the detailed methods given in refs. [4,5,13]); in the present study, a cutoff of $q < 0.2$ was used to denote statistically significant associations. HLA-associated polymorphisms were classified into two categories. "Nonadapted" amino acids are enriched in the absence of the restricting HLA of interest. Usually, "nonadapted" forms represent the consensus amino acid at that position, and they can be thought of as the "wild-type" or "susceptible" form particular to that allele. Conversely, "adapted" amino acids are those enriched in the presence of the HLA allele; these can be thought of as the escape variant particular to that allele.

3. Results and discussion

3.1. Genetic variability of the vpu gene

We successfully amplified DNAs encompassing the *vpu* region from 216 of 240 samples (90%). Firstly, we analyzed the amino acid variability at each codon of Vpu by determining its Shannon entropy score. Two amino acid residues, Trp23 and Arg49, showed highly conserved (>98%) among individual sequences. Instead, most codons displayed substantial variability, with the average of the entropy score reaching 0.58 (Fig. 1A), confirming the findings by Yusim et al., which showed that Vpu is a highly variable protein [10]. Also, the amino acid variability of each codon in the present study correlated strongly with that of published subtype B sequence data from the Los Alamos database (Fig. 1B), suggesting that our observed pattern of amino acid variation in Vpu was generally representative of the variation observed in HIV-1 subtype B. In fact, the consensus amino acid sequences of subtype B and the present dataset were identical except for 3 amino-acid residues: positions 3, 5, and 24 (Fig. 1C). These amino acid residues were highly variable (Fig. 1A) and not directly associated with known Vpu functions (Fig. 1C).

3.2. HLA-associated polymorphisms in Vpu

As HLA-I-mediated selective contributes to HIV-1 sequence variability, especially the accessory protein Nef [4], we sought to examine whether HLA-I-mediated selective pressure substantially influenced the evolution of Vpu, another accessory protein. We applied a phylogenetic dependency network model [13], which adjusts for the confounding effects of HIV phylogeny, HIV codon covariation and linkage disequilibrium of HLA-I alleles.

In our dataset of 216 individuals, we identified only three HIV-HLA associations in Vpu: a nonadapted association between C*03 and Glu-5, a nonadapted association between A*33:03 and Arg-37, and an adapted association between A*33:03 and Lys-37. The presence of both nonadapted and adapted A*33:03-associated polymorphisms at Vpu codon 37 is consistent with an Arginine-to-Lysine escape mutation occurring at the C-terminus of the immunodominant HLA-A*33:03-restricted epitope in Vpu, ^{29}EYRKILRQR^{37} [11]. However, there was no HLA-restricted T cell epitopes around Vpu position 5 have been reported. Although we might have missed some polymorphisms due to the limited sample size in this study, these data suggest that HLA-I-mediated selective pressure toward Vpu does not substantially drive Vpu variability at the population level in this cohort.

3.3. HLA-associated polymorphisms in alternating reading frames

CTLs can recognize epitopes encoded by alternate reading frames including the antisense-strand sequences of HIV-1 *gag*, *pol*, and *nef* [17,18]. Therefore, we also investigated HIV-HLA polymorphism associations in peptide sequences encoded by alternative reading frames of the *vpu* gene. We observed no statistically significant HLA-associated polymorphisms in alternate or antisense reading frames, except for a single HLA-B*40:01 associated "adapted" lysine polymorphism at codon 2 of the overlapping Envelope reading frame which is initiated in the middle of the *vpu* gene (ORF + 2; Table 1, Fig. 2). Although this association was between Lys-2 of Env and *HLA-B*40:01*, no CTL epitopes have been reported in the context of HLA-B*40:01 in this region. Using bioinformatic prediction programs Epipred [19] and BIMAS [20] we

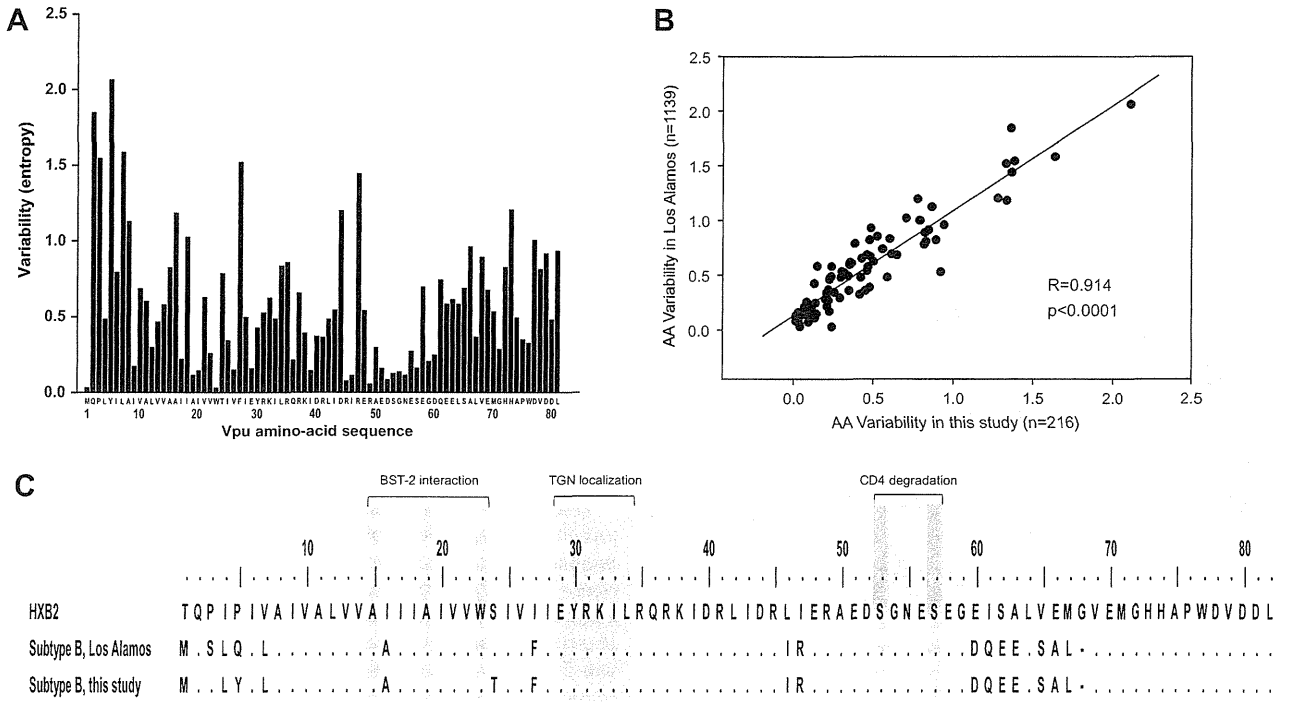

Fig. 1. Variability of the amino acid residues of HIV-1 Vpu. The amino acid sequence of Vpu was analyzed based on the cross-sectional studies on 216 HIV-infected subjects. The amino acid variability at each position of Vpu was analyzed by determining its Shannon entropy score (panel A). The Vpu sequence (subtype B, $n = 1139$) was retrieved from the Los Alamos HIV sequence database, analyzed for its amino acid variability, and compared with subtype B obtained from this study using Spearman Rank Order Correlation (panel B). The consensus sequences of Vpu obtained from Los Alamos database and this study were aligned with reference strain HXB2 and regions responsible for some key Vpu functions highlighted (panel C).

Table 1
Summary of HIV-HLA associations in the Vpu-encoded region.

RF	Protein	Pos HXB2	aa	HLA	Association	*p*-Value	*q*-Value	Known epitope	
								Sequence	Reference
+1	Vpu	5	E	C*03	Nonadapted	2.13×10^{-5}	1.52×10^{-1}	none	–
		37	R	A*22:03	Nonadapted	3.40×10^{-6}	5.50×10^{-2}	^{29}EYRKILRQR^{37}	[11]
		37	K	A*33:03	Adapted	2.80×10^{-5}	1.52×10^{-1}	^{29}EYRKILRQR^{37}	[11]
+2	Env	2	K	B*40:01	Adapted	1.63×10^{-5}	1.67×10^{-1}	none	–

RF, reading frame; Pos HXB2, amino acid position when aligned to HXB2 sequence.

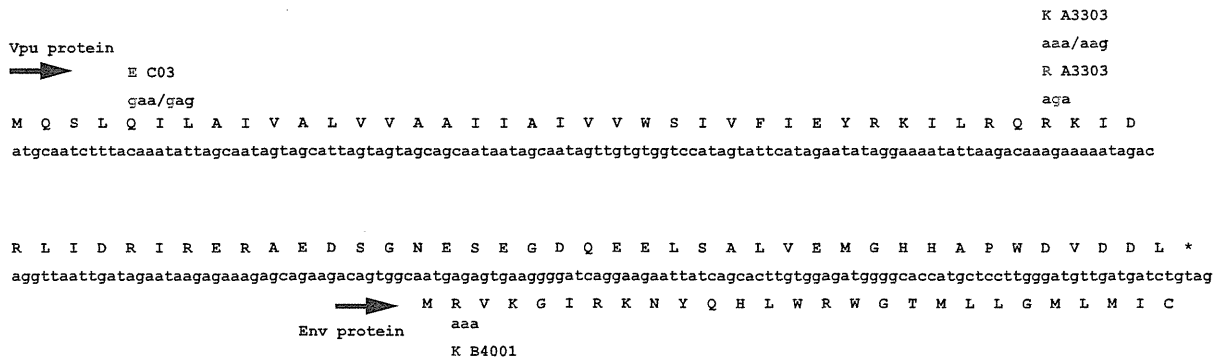

Fig. 2. The Vpu and a part of Env proteins and their associations with host HLA class I alleles. The nucleotide sequence and its deduced amino acid sequence of Vpu and of an overlapping part of Env with reference to the subtype B consensus sequence of Los Alamos database is shown. The amino acid residues associated with the indicated HLA class I alleles ($p < 0.05$, $q < 0.2$) are shown with adapted (red) and nonadapted (blue) residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

attempted to predict B*40:01-restricted CTL epitopes, but found none (data not shown). This failure is most likely due to the presence of several basic amino acids, such as Arg and Lys, in this region of Env (Fig. 2), as it has been shown that HLA-B*40:01

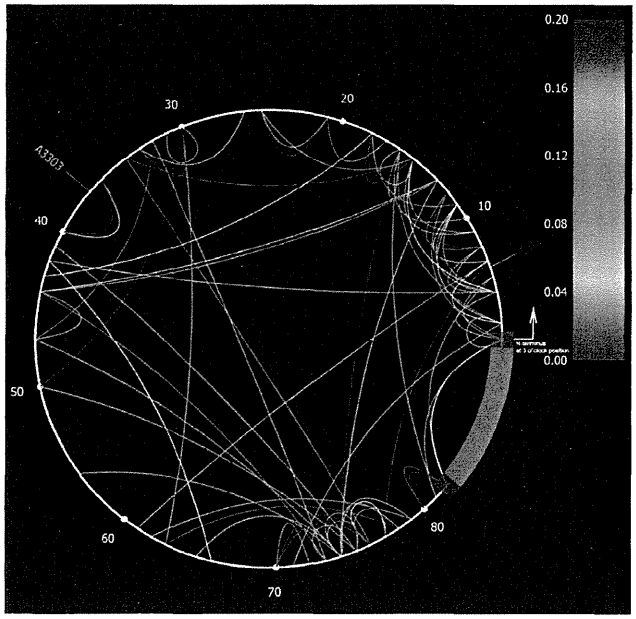

Fig. 3. Amino acid codon–codon covariation in Vpu. The circular map, generated by the PhyloDv software [13], shows Vpu codon–codon covariation associations as inner arcs connecting the association sites, with the HLA associations as tags pointing to their corresponding sites. Q values of individual codon pairs are represented as a heat map shown at the right.

preferentially binds peptides with acidic residues at their anchors [21]. This issue needs to be clarified in further studies using immunologic assays. Taken together, our results suggest that HLA-I-mediated selective pressure do not contribute to a large extent to population-level sequence variation in HIV-1 Vpu.

3.4. Codon–codon covariation of Vpu

Given that Vpu is functionally important in viral replication *in vivo*, the highly variable nature of Vpu amino acid sequences could be explained by complex networks of codon–codon covariation and/or secondary/compensatory mutation pathways. We therefore examined the codon–codon covariation of Vpu by using the phylogenetic dependency network model. Although Vpu consists of only 81 amino acids, we identified 103 covarying codon pairs in Vpu, displayed in Fig. 3. The covariation network in Vpu showed an uneven distribution, with a large number of codon–codon covariation networks at the N-terminal membrane-spanning region, a region responsible for BST-2 interaction [22]. Interestingly, the 3 HIV-HLA associations (Table 1, Fig. 2) were not significantly linked to any other amino acid residues. These data suggest that the conformation and function of Vpu may be preserved through many possible combinations of primary and secondary polymorphisms and that the HLA-I-associated immune-mediated selective pressure may have only a minor effect on such Vpu polymorphisms.

3.5. Association between Vpu polymorphisms and clinical parameters

Finally, we explored associations between Vpu polymorphisms and clinical parameters of HIV-infected patients (i.e., CD4 counts and plasma viral load). We observed no significant associations between Vpu polymorphisms and CD4 counts. However we identified a statistically significant association between amino acid residues at position 5 and viral load (Fig. 4). The patients harboring Val at Vpu-5 had significantly higher viral loads compared to those with amino acid residues other than Val at this position. Thus, amino acid residues at position 5 of Vpu showed several interesting features, i.e., the highest variability of all Vpu amino acids (Fig. 1A), nonadapted association of Glu-5 with *HLA-Cw*03*, and association of Val-5 with the increased viral load. Considering that the amino acid residue at this position is located in close proximity to the membrane-spanning region and that this region is functionally important for BST-2 binding, it would be interesting to examine functional effects of amino acid polymorphisms at position 5, whether they are mediated by host immune responses or otherwise.

In summary, we report here comparably fewer HLA-associated mutations in Vpu in this cohort although host HLA class I allele-associated immune responses are major forces driving the evolution of HIV-1 accessory proteins, such as Nef. Taken together, we conclude that the influence of immune selection on evolution of Vpu

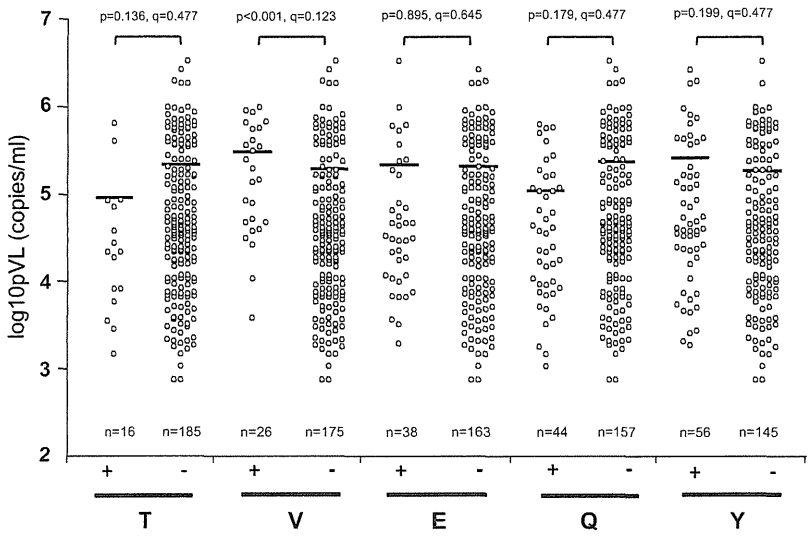

Fig. 4. Association between plasma viral load and amino acid polymorphism at position 5 of Vpu. HIV plasma viral loads, stratified by amino acid expression at Vpu codon 5, are shown. Vpu codon 5 exhibited 11 different amino acids positioning in our dataset; only those observed in >10 patients are shown here. Horizontal bars indicate medians. Statistical analysis was performed using the Mann-Whitney U-test.

at the population level may be reduced compared to other highly variable HIV-1 proteins, providing us with additional insight into differential evolutional pathways among viral accessory proteins.

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Anti-Human Immunodeficiency Virus Type 1 Activity of Novel 6-Substituted 1-Benzyl-3-(3,5-Dimethylbenzyl)Uracil Derivatives

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Nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs) are important components of current combination therapies for human immunodeficiency virus type 1 (HIV-1) infection. In screening of chemical libraries, we found 6-azido-1-benzyl-3-(3,5-dimethylbenzyl)uracil (AzBBU) and 6-amino-1-benzyl-3-(3,5-dimethylbenzyl)uracil (AmBBU) to be highly active and selective inhibitors of HIV-1 replication *in vitro*. To determine the resistance profiles of these compounds, we conducted a long-term culture of HIV-1-infected MT-4 cells with escalating concentrations of each compound. After serial passages of the infected cells, escape viruses were obtained, and they were more than 500-fold resistant to the uracil derivatives compared to the wild type. Sequence analysis was conducted for RT of the escape viruses at passages 12 and 24. The amino acid mutation Y181C in the polymerase domain of RT was detected for all escape viruses. Docking studies using the crystal structure of RT showed that AmBBU requires the amino acid residues Leu100, Val106, Tyr181, and Trp229 for exerting its inhibitory effect on HIV-1. Four additional amino acid changes (K451R, R461K, T468P, and D471N) were identified in the RNase H domain of RT; however, their precise role in the acquisition of resistance is still unclear. In conclusion, the initial mutation Y181C seems sufficient for the acquisition of resistance to the uracil derivatives AzBBU and AmBBU. Further studies are required to determine the precise role of each mutation in the acquisition of HIV-1 resistance.

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is responsible for synthesizing a double-stranded integrative cDNA from the single-stranded viral genomic RNA in the early virus life cycle. HIV-1 RT is a heterodimer composed of two subunits, p66 and p51, and p51 is generated by the proteolytic processing and removal of C terminus (amino acids 441 to 560) from p66 (32). The p66 subunit is composed of two spatially distinct domains: polymerase (residues 1 to 426) and RNase H (residues 427 to 560). The polymerase domain is composed of four subdomains: fingers (residues 1 to 85 and 118 to 155), palm (residues 86 to 117 and 156 to 236), thumb (237 to 318), and connection (319 to 426) (46). The polymerase domain creates a copy of the viral genome, while the RNase H domain promotes RNA degradation from the DNA/RNA duplex during reverse transcription. Other RNase H functions include the removal of $tRNA_3^{Lys}$, and the polypurine tract, which are primers for minus- and plus-strand DNA synthesis (11, 47, 58).

The polymerase domain is currently targeted by two classes of antiretroviral drugs, nucleoside RT inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs). NNRTIs are important components of current antiretroviral therapies for HIV-1. To date, more than 50 structurally diverse classes of compounds have been identified as genuine NNRTIs (60). Earlier NNRTIs include nevirapine (NVP), delavirdine (DLV), and efavirenz (EFV), and recent NNRTIs include etravirine (ETR). Several other NNRTIs, such as emivirine (MKC-442), underwent clinical trials, and yet they were not approved due to unfavorable pharmacokinetics, insufficient efficacy, and/or safety concerns (50). Recently, rilpivirine (RPV; formerly TMC278) has been formally licensed for clinical use in treatment-naive adult patients (56), while other NNRTIs, including IDX899, RDEA-428, and lersivirine, are currently under clinical development (1, 12, 30).

Although NNRTIs are generally well tolerated, a major limitation for all currently available NNRTIs is the low genetic barrier to resistance, which allows rapid emergence of drug resistance caused by a small number of amino acid mutations in the target region. HIV-1 drug resistance mutations in RT are extensively characterized for NRTIs and NNRTIs (29). NNRTIs inhibit HIV-1 by allosteric binding to a hydrophobic pocket in the RT about 10 Å behind the catalytic site (48). The positions associated with NNRTI resistance that make up the central NNRTI binding pocket are L100, K101, K103, V106, V108, V179, Y181, Y188, G190, F227, and W229. Additional positions that make up the pocket include E138, which is contributed by the p51 subunit, and M230, L234, P236, K238, and Y318, which form part of an extended pocket. Additional accessory NNRTI resistance abutting positions that form the NNRTI binding pocket include A98 and P225.

Mutations that are selected after failure of treatment with NNRTIs are located in the enzyme hydrophobic pocket, and they reduce the binding affinity of the inhibitors to the enzyme (7). A single mutation in the NNRTI-binding pocket may confer high-level resistance to one or more NNRTIs. Since NVP, DLV, and EFV have similar binding modes to RT, viruses resistant to one of these compounds develop cross-resistance to the others. ETR maintains its activity against NVP-, DLV-, or EFV-resistant mutants due to its ability of multiple binding modes to RT.

In order to improve the design of novel NNRTIs for future clinical development, *in vitro* isolation and analyses of drug-resis-

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tant viruses are necessary to obtain valuable information on the resistance patterns of novel compounds. We previously synthesized and evaluated nine novel uracil analogues as NNRTIs, including four 1-substituted 3-(3,5-dimethylbenzyl)-5-fluorouracils and five 6-substituted 1-benzyl-3-(3,5-dimethylbenzyl)uracils (28). Two of these compounds—6-azido-1-benzyl-3-(3,5-dimethylbenzyl)uracil (AzBBU) and 6-amino-1-benzyl-3-(3,5-dimethylbenzyl) uracil (AmBBU)—were found to be highly active and selective inhibitors of HIV-1 replication *in vitro*.

In the present study, we conducted a long-term culture experiment with HIV-1-infected MT-4 cells with escalating concentrations of AzBBU and AmBBU. After serial passages of the infected cells, escape viruses were obtained, which displayed complete resistance to these compounds. Sequence analysis of RT from escape viruses was performed to determine the mutations related to the acquisition of resistance.

MATERIALS AND METHODS

Compounds. AzBBU and AmBBU were synthesized as previously described (28). The lead compound 1-benzyl-3-(3,5-dimethylbenzyl) uracil (BBF-29), 6-benzyl-1-ethoxymethyl-5-isopropyluracil (MKC-442), and the nucleoside analog 2′,3′-didehydro-3′-deoxy-4′-ethynylthymidine (4′-Ed4T) were prepared according as previously described (25, 34, 52). All compounds were dissolved in dimethyl sulfoxide at 100 mM and stored at −20°C until use. The chemical structures of AzBBU, AmBBU, BBF-29, MKC-442, and 4′-Ed4T are shown in Fig. 1.

Cells and viruses. MT-4 and M8166 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U of penicillin G/ml, and 100 μg of streptomycin/ml. The HIV-1 strain III_B, the HIV-1 resistance strain $III_{B\text{-}R}$, and the HIV-2 strain ROD were used throughout the antiviral experiments (Table 1). $III_{B\text{-}R}$ is a NNRTI-resistant mutant established by a serial passage of infected cells in the presence of increasing concentrations of MKC-442 (5). Viruses were propagated and titrated in MT-4 cells (HIV-1) or M8166 cells (HIV-2). Virus stocks were stored at −80°C until use. Escape viruses obtained after long-term culture with AzBBU and AmBBU were used for the following anti-HIV-1 experiments (Table 2).

Antiviral assays. The antiviral activity of test compounds against HIV-1 and HIV-2 was determined by the inhibition of virus-induced cytopathic effect (CPE) (4). Briefly, MT-4 or M8166 cells (10^5 cells/ml) were infected with HIV-1 or HIV-2, respectively, at a multiplicity of infection (MOI) of 0.1 and were cultured in the presence of various concentrations of the tested compounds. After a 4-day incubation at 37°C, the number of viable cells was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method (39). The cytotoxicity of the compounds was evaluated in parallel with their antiviral activity, based on the viability of mock-infected cells, as determined by the MTT method. All experiments were performed in triplicate.

Long-term culture of infected MT-4 cells. MT-4 cells were infected with the HIV-1 strain III_B and incubated at 37°C for 4 days in the presence of compounds. The initial concentration of AzBBU and AmBBU corresponded to 2-fold higher than their 50% effective concentrations (EC_{50}s), i.e., 0.175 and 0.119 μM, respectively. As control cultures, exactly identical passages of the infected MT-4 cells in the absence of the compounds were carried out in parallel with the cultures exposed to the compounds. At each passage, virus-induced CPE of the cells was monitored to confirm virus replication. The concentration of each compound was escalated 2-fold, when the CPE in the compound-treated culture exceeded 70%. The escape viruses as well as the control viruses were propagated once in MT-4 cells, and they were used for further experiments.

Sequence analysis of RT. Genomic DNA was extracted from the infected MT-4 cells with a DNA extraction kit (Wako, Tokyo, Japan). The extracted DNA was quantified using a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE) and subjected to nested PCR. Two regions of RT were amplified (RT1 and RT2). The first PCR consisted of an initial denaturation at 95°C for 2 min, followed by 40 cycles (95°C for 1 min, 46°C for 1 min, and 72°C for 1 min) and a final extension at 72°C for 5 min. The primers rt-1-f (5′-AGGGGGAATTGG AGGTTT-3′) and rt-1-r (5′-TCCCACAACTTCTGTATGTC-3′) were used to amplify the region RT1, and the primers rt-2-f (5′-ATGAACTCC ATCCTGATAAATG-3′) and rt-2-r (5′-TGTACAATCTAGTTGCCATA T-3′) were used to amplify the region RT2. The second PCR consisted of an initial denaturation at 95°C for 2 min, followed by 40 cycles (95°C for 1 min, 48°C for 1 min, and 72°C for 1 min) and a final extension at 72°C for 5 min. The primers rt-12-f (5′-CCAGTAAAATTAAAGCCAG-3′) and rt-12-r (5′-TCCCACTAACTTCTGTATGTC-3′) were used to amplify the region RT1, and the primers rt-22-f (5′-CCAGAAAAAGACAGCTG GACT-3′) and rt-22-r (5′-TGGCAGGTTAAAATCACTAGCC-3′) were used to amplify the region RT2. The second PCR generated fragments encompassing nucleotides 2120 to 2881 (RT1) and nucleotides 2834 to 3862 (RT2) of the RT gene corresponding to the HIV-1 complete genome (GenBank accession number AF033819). The amplified products were confirmed by capillary gel electrophoresis using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). The PCR products were sequenced directly with a cycle sequence kit BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA), using both forward and reverse primers on an automated DNA analyzer model 3730 (Applied Biosystems), according to the manufacturer's instructions.

Docking study of compounds against HIV-1 RT. All *in silico* studies were performed using Molecular Operating Environment (MOE) soft-

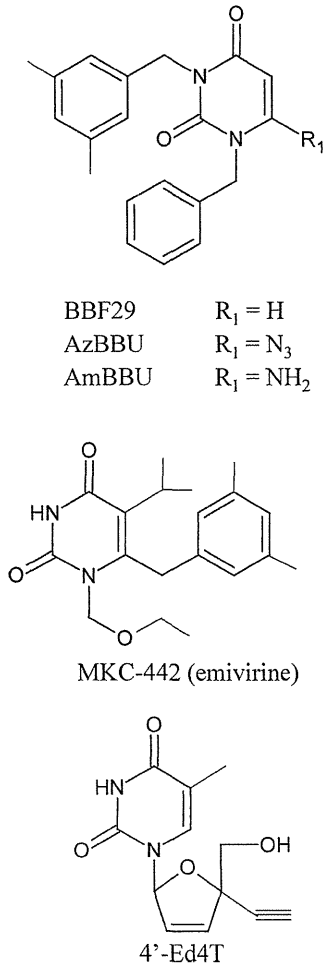

FIG 1 Structures of BBF-29, AzBBU, AmBBU, MKC-442, and 4′-Ed4T.

TABLE 1 Antiviral activity of AzBBU and AmBBU against HIV-1 and HIV-2

Compound	Antiviral activity (mean EC_{50} [μM] and CC_{50} [μM] ± SD)[a] against:					
	HIV-1 III_B		HIV-1 III_{B-R}		HIV-2 ROD	
	EC_{50}	CC_{50}	EC_{50}	CC_{50}	EC_{50}	CC_{50}
AzBBU	0.088 ± 0.009	40.5 ± 6.7	>45.1	45.1 ± 0.5	>40.0	40.0 ± 2.7
AmBBU	0.060 ± 0.011	50.1 ± 1.1	>50.5	50.5 ± 6.3	>45.9	45.9 ± 1.2
BBF-29	0.26 ± 0.02	43.2 ± 9.1	13.9 ± 6.0	>100	>100	>100
MKC-442	0.015 ± 0.002	>100	6.2 ± 1.4	>100		
NVP	0.057 ± 0.005	>100	52.1 ± 23.6	>100		
4′-Ed4T	0.029 ± 0.008	>100	0.053 ± 0.038	>100	0.019 ± 0.007	>100

[a] EC_{50}, 50% effective concentration that inhibits virus-induced CPE of infected cells by 50%; CC_{50}, 50% cytotoxic concentration that reduces the viability of uninfected cells by 50%.The data represent the means for three independent experiments.

ware (Chemical Computing Group, Montreal, Quebec, Canada). The X-ray crystal structure of HIV-1 RT (PDB code 3m8p) (40) was downloaded from PDB at the Research Collaboration for Structural Bioinfomatics (http://www.rcsb.org/pdb/home/home.do) and optimized for the docking study by removing ligand and water, adding hydrogen atoms, assigning atomic charges, and minimizing using the Merck molecular force field 94X (MMFF94x) (22, 23, 55). Based on this structure, the structure of the HIV-1 RT (Y181C) was constructed. The docking site at the HIV-1 RT structure was searched by Alpha Site Finder, a function of MOE. Partial charges were added to the compound and a maximum of 250 conformers were generated using MMFF94x. MOE-ASEDock 2005 (Ryoka Systems, Tokyo, Japan) was then used for the docking of the compound to HIV-1 RT, and docking scores were calculated (19).

Statistical analysis. Statistical analysis for the EC_{50}s of the test compounds against the wild-type and resistant viruses was performed using an unpaired two-tailed Student *t* test. *P* values of <0.01 were considered statistically significant.

RESULTS

Antiviral activity of AzBBU and AmBBU against HIV-1 and HIV-2. AzBBU and AmBBU were tested for their inhibitory effects on the replication of HIV-1 III_B, HIV-1 III_{B-R}, and HIV-2 ROD. The NNRTIs BBF-29, MKC-442, and NVP, as well as the nucleoside analog 4′-Ed4T, were also tested for comparison. Their activities are given in Table 1. AzBBU and AmBBU showed high activity against HIV-1 III_B with similar EC_{50}s (0.088 ± 0.009 and 0.060 ± 0.011 μM, respectively) and 50% cytotoxic concentrations (CC_{50}s) (40.5 ± 6.7 and 50.1 ± 1.1 μM, respectively). AmBBU showed a higher selectivity index (SI) than AzBBU (SI = 835 versus SI = 460). These results are in accordance with those in the previous report (28). Although these compounds showed higher anti-HIV-1 activity against III_B compared to the lead compound BBF-29 (0.26 ± 0.02 μM), they were not active against the NNRTI-resistant HIV-1 stain III_{B-R}. In addition, AzBBU and AmBBU did not show any activity against HIV-2 ROD. In contrast, NRTI 4′-Ed4T was equally active against HIV-1 III_B, HIV-1 III_{B-R}, and HIV-2 ROD.

Isolation of escape viruses. Long-term cultures of HIV-1 (III_B strain)-infected MT-4 cells were started in the absence or presence of AzBBU and AmBBU (Fig. 2). The concentration of each compound was escalated 2-fold, when the CPE in the compound-treated culture exceeded 70%. At passage 24, the concentrations of AzBBU and AmBBU could reach 256-fold their EC_{50}s (22.4 and 15.2 μM, respectively). Viruses were isolated from culture supernatants at passages 12 and 24 (Fig. 2, points a, b, c, and d) and subjected to phenotypic and genotypic analyses.

Anti-HIV-1 activity of AzBBU and AmBBU against escape viruses. When AzBBU and AmBBU were examined for their activity against the escape viruses obtained at passages 12 and 24, the compounds did not show any significant inhibition at their nontoxic concentrations (Table 2). Thus, the isolates were more than 500-fold resistant to AzBBU and AmBBU compared to the wild type. The lead compound BBF-29 was also inactive against the escape viruses. The viruses had partial cross-resistance to MKC-442, probably due to its structural similarity (Fig. 1 and Table 2). NVP marginally inhibited the replication of the escape viruses. In contrast, 4′-Ed4T was equally inhibitory to the replication of the escape viruses and the wild type (Table 2).

TABLE 2 Anti-HIV-1 activity of AzBBU and AmBBU against escape viruses

Compound	Anti-HIV-1 activity (mean EC_{50} [μM] and CC_{50} [μM] ± SD)[a]							
	Passage 12				Passage 24			
	III_{B-AZ12}		III_{B-AM12}		III_{B-AZ24}		III_{B-AM24}	
	EC_{50}	CC_{50}	EC_{50}	CC_{50}	EC_{50}	CC_{50}	EC_{50}	CC_{50}
AzBBU	>44.5* (>500)	44.5 ± 0.3	>42.6* (>480)	42.6 ± 3.0	>43.6* (>500)	43.6 ± 0.5	>44.0* (>500)	44.0 ± 1.0
AmBBU	>60.7* (>1,000)	60.7 ± 6.5	>56.2* (>940)	56.2 ± 5.7	>48.1* (>800)	48.1 ± 4.8	>47.0* (>780)	47.0 ± 7.6
BBF-29	>46.4* (>180)	46.4 ± 9.5	>42.5* (>160)	42.5 ± 3.9	>49.4* (>190)	49.4 ± 3.0	>45.9* (>170)	45.9 ± 7.2
MKC-442	3.8 ± 1.2* (257)	>100	2.5 ± 0.6* (168)	>100	5.4 ± 3.5 (369)	>100	3.8 ± 1.2* (257)	>100
NVP	49.9 ± 10.9* (875)	>100	32.6 ± 4.6* (572)	>100	37.2 ± 3.5* (652)	>100	33.6 ± 6.7* (589)	>100
4′-Ed4T	0.038 ± 0.029 (1.3)	>100	0.092 ± 0.011* (3.2)	>100	0.028 ± 0.012 (1)	>100	0.023 ± 0.003 (0.8)	>100

[a] EC_{50}, 50% effective concentration that inhibits virus-induced CPE of infected cells by 50%; CC_{50}, 50% cytotoxic concentration that reduces the viability of uninfected cells by 50%. Fold changes, based on the EC_{50} for the wild type, are indicated in parentheses. The data represent the means for three independent experiments. Statistical analysis (Student *t* test) was performed for each EC_{50} compared to that of the wild type (*, $P < 0.01$).

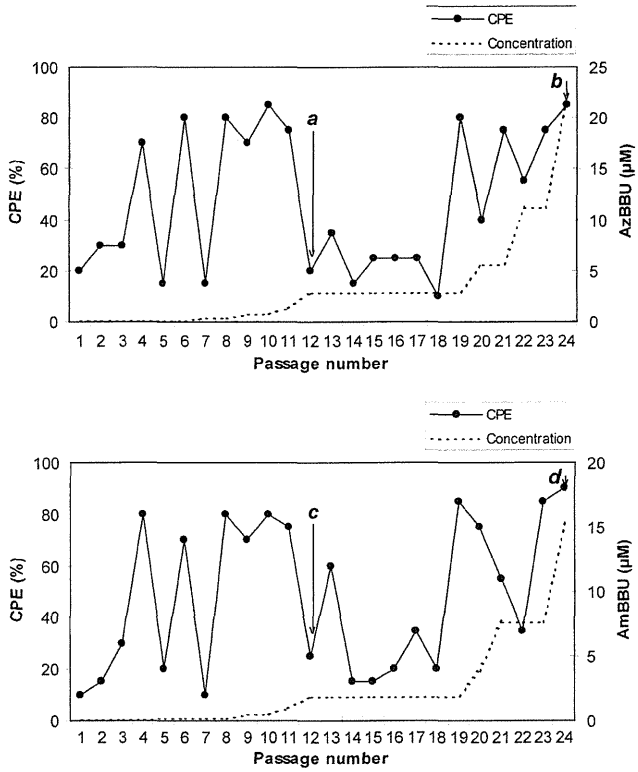

FIG 2 Long-term culture of infected MT-4 cells with escalating concentrations of AzBBU and AmBBU. MT-4 cells were infected with HIV-1 (III_B strain) and passaged every 4 days. Viral replication was monitored by determining the CPE of the cells at each passage. Culture supernatants from passages 12 and 24 were used for further experiments (CPE > 70%). Isolated viruses (points a, b, c, and d) were subjected to phenotypic and genotypic analyses. Points: a, passage 12, AzBBU ($III_{B\text{-}AZ12}$); b, passage 24, AzBBU ($III_{B\text{-}AZ24}$); c, passage 12, AmBBU ($III_{B\text{-}AM12}$); d, passage 24, AmBBU ($III_{B\text{-}AM24}$).

Amino acid changes of escape viruses. To determine what amino acid changes are associated with resistance to AzBBU and AmBBU, sequence analysis of full-length RT genes from escape viruses ($III_{B\text{-}AZ12}$, $III_{B\text{-}AM12}$, $III_{B\text{-}AZ24}$, $III_{B\text{-}AM24}$), as well as the wild-type control without treatment (III_B), was further performed at passages 12 and 24. The sequences of the escape viruses, III_B-AzBBU and III_B-AmBBU, were deposited in a public database (GenBank accession numbers JQ070415 and JQ070416). In addition, the sequence of the resistant strain $III_{B\text{-}R}$ was also analyzed for comparison (GenBank accession number JQ070417). Figure 3 shows the RT (subunit p66) amino acid sequences of escape viruses. Several synonymous mutations were observed in the RT gene. Overall, 5 amino acid changes were observed. One was in the polymerase domain, and four were in the RNase H domain. The same amino acid changes were detected at passages 12 and 24 (Fig. 3). Mutation Y181C in the polymerase domain was identified in all escape viruses. The resistant strain $III_{B\text{-}R}$ displayed the mutation V108I in addition to Y181C. Four additional amino acid changes (K451R, R461K, T468P, and D471N) were detected in the RNase H domain of all escape viruses.

Docking studies of AmBBU. Docking of the metabolically relevant derivative AmBBU to the binding-pocket of HIV-1 RT was performed. Figure 4 shows the proposed interactions between HIV-1 RT and AmBBU for HIV-1 RT wild type (Fig. 4A) and the mutant Y181C (Fig. 4B). AmBBU interacted with the amino acid residues Leu100, Val106, Tyr181, and Trp229 of HIV-1 RT (wt) through arene-H interactions (arene-H) (Fig. 4A), such as the hydrogen in the side chain of Leu100 with the central benzene ring (2-pyrimidine) of AmBBU, the hydrogen in the side chain of Val106 with 1-benzyl of AmBBU, the phenyl ring in the side chain of Tyr181 with hydrogen (3-methyl) at 3-(3,5-dimethylbenzyl) of AmBBU, and the indol ring in the side chain of Trp229 with 4′-hydrogen of 3-(3,5-dimethylbenzyl) of AmBBU (D2 representation in Fig. 4A). The compound lost the interaction at position 181 when replaced by cysteine (Y181C) (Fig. 4B). According to these data, the docking score between HIV-1 RT (wt) and AmBBU (−13.5110 kcal/mol) was higher than that between HIV-1 RT (Y181C) and AmBBU (−11.1648 kcal/mol).

DISCUSSION

Initially, we evaluated the antiviral activities of AzBBU and AmBBU against the HIV-1 strains III_B and $III_{B\text{-}R}$ and the HIV-2 strain ROD. AzBBU and AmBBU were highly active against III_B, and the EC_{50}s were similar for both compounds (Table 1). This is explainable, since the 6-azido uracil derivative (AzBBU) may be reduced metabolically to its 6-amino congener (AmBBU) in cell cultures (28). These compounds showed higher activity against III_B than the 1-substituted 3-(3,5-dimethylbenzyl)uracils previously reported (33). In contrast, AzBBU and AmBBU were not active against $III_{B\text{-}R}$, although the lead compound BBF-29 had weak activity. This may be due to the azido and amino groups introduced at 6-position of the 1-benzyl moiety, which is not present in BBF-29 (Fig. 1). Thus, 6-azido and 6-amino substitutions on the uracil ring increased the antiviral activity against III_B but decreased the antiviral activity against $III_{B\text{-}R}$. In addition, AzBBU and AmBBU did not show any activity against HIV-2 ROD. This is consistent with previous reports showing that HIV-2 is intrinsically resistant to most NNRTIs (14, 57). As expected, the nucleoside analog 4′-Ed4T was almost equally active against HIV-1 III_B, $III_{B\text{-}R}$, and HIV-2 ROD.

We isolated two HIV-1 strains highly resistant to AzBBU and AmBBU through long-term culture of HIV-1 III_B-infected MT-4 cells (Fig. 2). The phenotypic analysis revealed that escape viruses had partial cross-resistance to MKC-442, probably due to its structural similarity (Table 2 and Fig. 1). A similar finding has been reported for 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) derivatives, where the HEPT-resistant virus also displayed cross-resistant to virtually all of other HIV-1-specific inhibitors, including NVP (6). 4′-Ed4T was equally active against the AzBBU- and AmBBU-resistant viruses compared to the wild type because of its different mechanism of action.

Our genotypic analysis revealed amino acid changes associated with resistance to AzBBU and AmBBU (Fig. 3). Several mutations were observed in the RT gene of resistant viruses, but some of them corresponded to synonymous mutations (Fig. 3). Amino acid changes were accumulated within a short period of cultivation (from passage 12, 48 days). Five amino acid changes were identified in all escape viruses at passages 12 and 24 (Fig. 3). They were attributable to the selection pressure by the compounds and were not the consequences of *in vitro* passage of infected cells, since these changes could be identified only for the escape viruses but not for the corresponding control viruses (data not shown).

We firstly identified the mutation Y181C within the polymerase domain of RT. Mutations responsible for NNRTI resistance

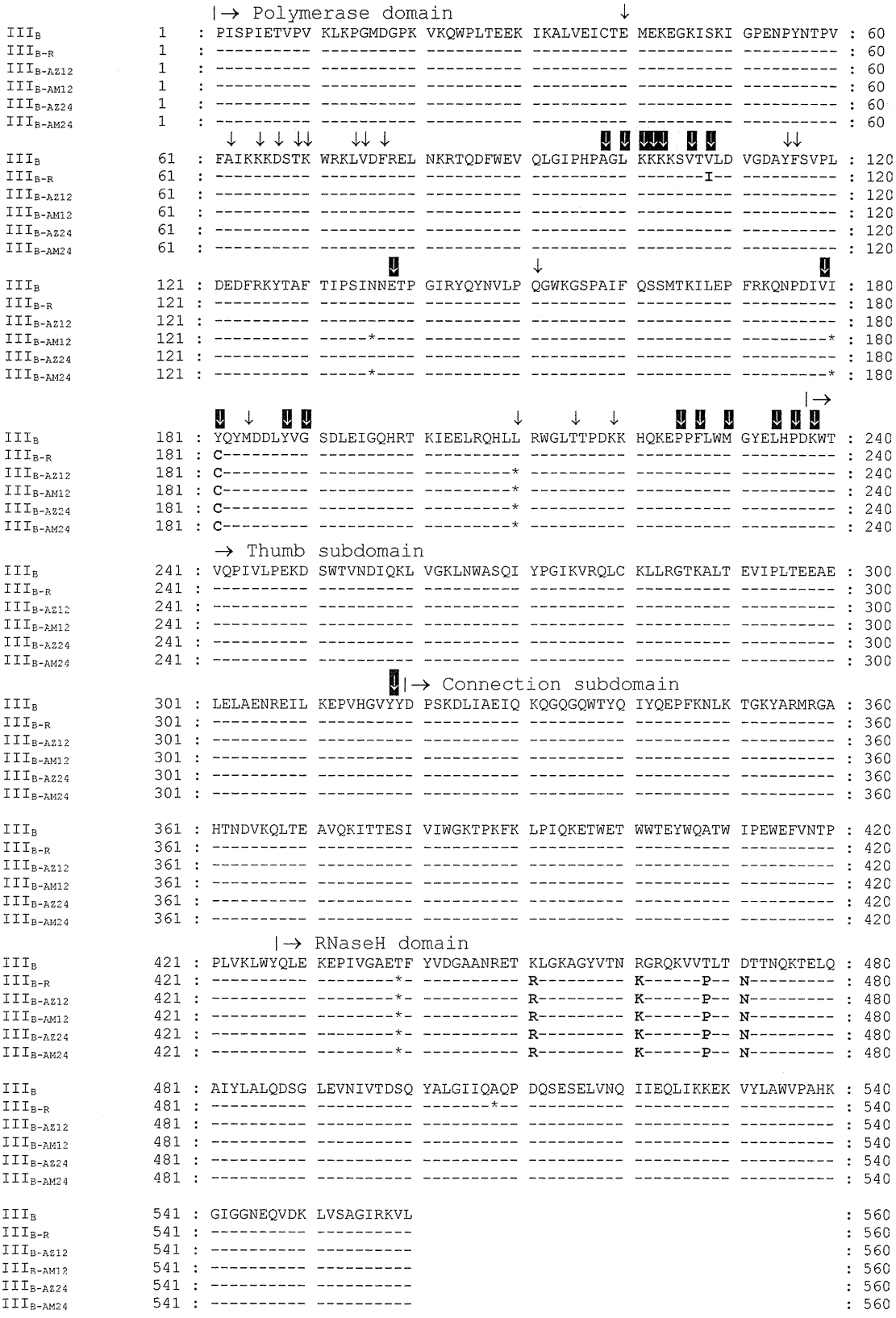

FIG 3 RT (subunit p66) amino acid sequences of the escape viruses. The control sequence without treatment was used as reference (WT). Asterisks (*) indicate the positions for synonymous mutations. Black arrows indicate common NRTI resistance mutations. White arrows indicate common NNRTI resistance mutations.

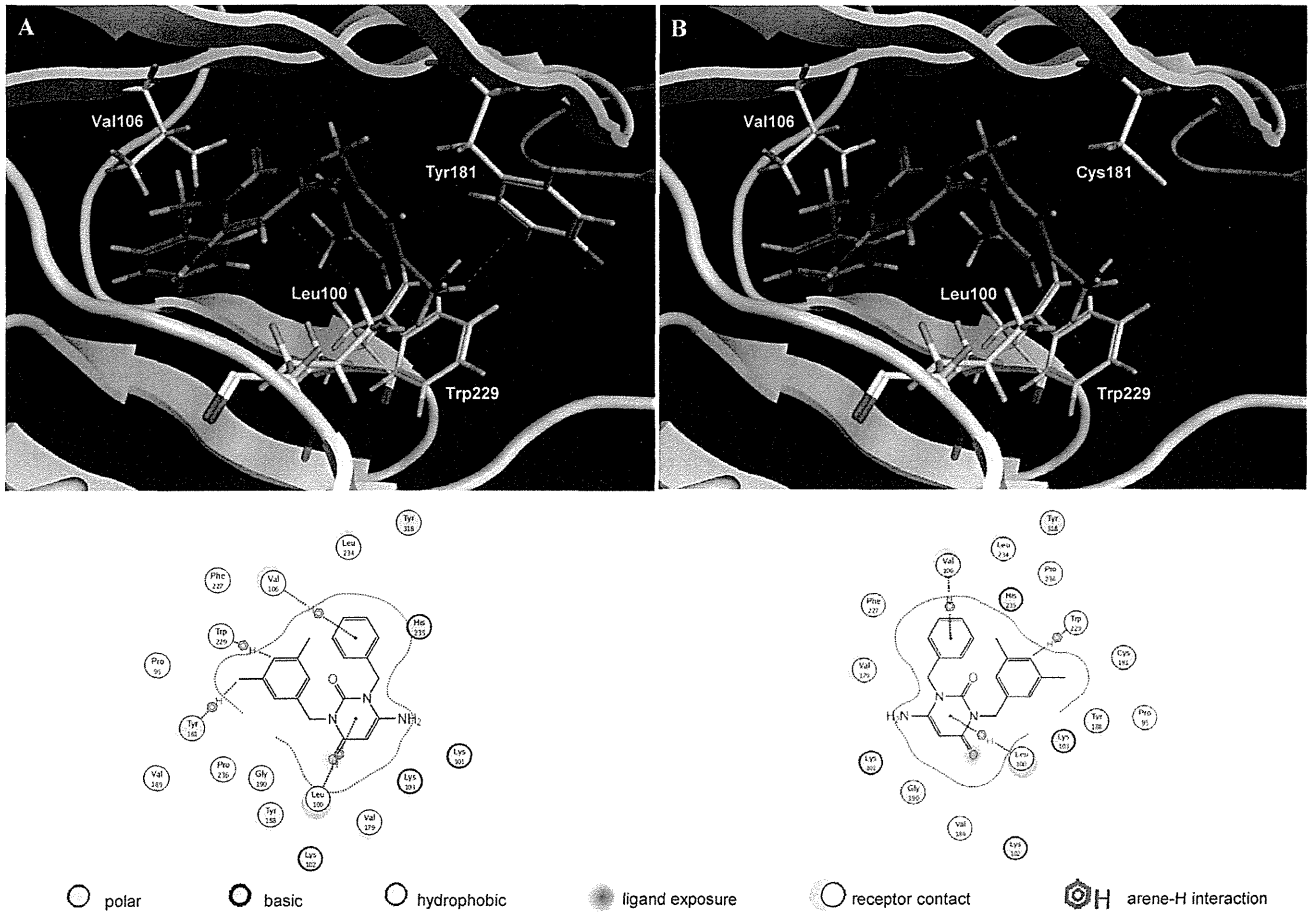

FIG 4 Docking of AmBBU to the binding pocket of HIV-1 RT. Docking structures between HIV-1 RT and AmBBU are shown for HIV-1 RT wt (A) and the mutant Y181C (B). The backbone is represented by white ribbons, and the side chains (residues 100, 106, 181, and 229) are represented by colored wire style. AmBBU is represented by magenta wire style. Dotted lines show the interactions between HIV-1 RT and AmBBU. A D2 representation of the interactions is presented below the docking.

occur in the hydrophobic inhibitor-binding pocket of RT, which includes the residue Y181 (46). A single mutation in this pocket can lead to high-level resistance to earlier NNRTIs, including EFV and NVP. However, two or more mutations are required to cause high-level resistance to ETR, RPV, and other recent NNRTIs (3, 35, 53, 61). Y181C alone can cause multidrug resistance to NNRTIs, such as high-level resistance to NVP and DLV, and low-level resistance to EFV (18). In addition, Y181C provides the mutational foundation for the development of higher levels of ETR resistance (49). The Y181C mutation has emerged as an initial mutation in previous studies in patients failing an NNRTI-containing regimen, including NVP monotherapy (15, 43) and DLV monotherapy (17). K103N is frequently observed among NNRTI resistance mutations, but it is usually followed by Y181C and G190A (27). Resistance to HEPT derivatives is generated by mutations at multiple sites in the HIV-1 RT (8), while 1-(3-cyclopenten-1-yl)methyl-6-(3,5-dimethylbenzoyl)-5-ethyl-2,4-pyrimidinedione (SJ-3366) selected for a virus with the mutation Y181C after five tissue culture passages (9). In the present study, the initial mutation Y181C was sufficient for the acquisition of HIV-1 resistance to AzBBU and AmBBU. Other common NNRTI resistance mutations, such as K101E, K103N, and Y188C, were not identified.

Using docking studies, we examined the binding sites for the metabolically relevant derivative AmBBU on the allosteric pocket of HIV-1 RT (Fig. 4). According to the docking, AmBBU binds the allosteric pocket through arene-H interactions with the amino acid residues Leu100, Val106, Tyr181, and Trp229 (Fig. 4A). When the RT mutant Y181C was used for docking, AmBBU lost the interaction at position 181 (Fig. 4B). The docking score between HIV-1 RT (wt) and AmBBU was higher than that between HIV-1 RT (Y181C) and AmBBU, indicating the importance of the residue Tyr181 in the binding to the allosteric pocket. Most NNRTIs are engaged in the H-bond with the backbone of the residues Lys101 and/or Lys103 of RT (13, 26, 41). Previous docking studies suggested an H-bond between the amide of Lys101 and nitrogen of the cyanomethyl and picolyl group of 1-substituted 3-(3,5-dimethylbenzyl)uracils (33). In our previous study, we showed that 6-subtitutions on the uracil ring resulted in elevation of the anti-HIV-1 activity of the uracil derivatives (28). The structure-activity relationship among these uracil derivatives suggested that the strong anti-HIV-1 activity of the 6-amino derivative AmBBU is due to the H-bond formed between the 6-amino group of AmBBU and the amide group of the residue Lys101, as well as hydrophobic interactions (28). Here, our docking showed

TABLE 3 NNRTI-resistant mutations in the polymerase domain and amino acid changes in the RNase H domain of escape virus

		RT domain[a]								
		Polymerase					RNase H			
GenBank accession no.	Name	A98	K102	K103	V179	Y181	K451	R461	T468	D471
A04321	IIIB LAI	–	–	–	–	–	–	–	P	–
A07867	LAI-J19	–	–	–	–	–	–	–	–	–
AB221005	Ba-L	Q	–	–	–	–	–	–	S	–
AF033819	HXB2_copy LAI	–	–	–	–	–	–	–	–	–
AF070521	NL43E9 LAI IIIB_NY5	–	Q	–	–	–	–	–	P	–
D86068	MCK1 LAI IIIB	–	–	–	–	–	R	K	P	N
D86069	PM213 LAI IIIB	–	–	–	–	–	–	–	–	–
EU541617	pIIIB	–	Q	–	–	–	–	–	P	–
HB388803	MN patent seq	–	–	–	–	–	–	–	S	–
K02007	SF2 LAV2 ARV2	–	–	–	–	–	–	–	S	–
K02013	LAI BRU	–	–	–	–	–	–	–	–	–
K02083	PV22 LAI IIIB	–	–	–	–	–	R	K	P	N
K03455	HXB2-LAI-IIIB-BRU	–	–	–	–	–	–	–	–	–
M17449	MNCG MN	–	–	–	–	–	–	–	S	–
NC_001802	HXB2-LAI-HXB2R	–	–	–	–	–	–	–	–	–
U26942	NL4_3 LAI_NY5 pNL43 NL43	–	Q	–	–	–	–	–	P	–
U63632	JRFL JR_FL	–	–	R	I	–	–	–	S	–
X01762	REHTLV3 LAI IIIB	–	–	–	–	–	–	K	P	N
JQ070415	AzBBU IIIB-AZ	–	–	–	–	C	R	K	P	N
JQ070416	AmBBU IIIB-AM	–	–	–	–	C	R	K	P	N

[a] The default amino acids and position numbers are specified in each column subheading. –, No change.

arene-H interactions of AmBBU with Leu100, Val106, Tyr181, and Trp229 (Fig. 4A). However, we could not identify the precise role of the 6-amino substitution in the binding to the allosteric pocket and how it leads to an increased anti-HIV-1 activity. Thus, although several interactions occur within the allosteric pocket, the interaction with the Tyr181 residue appears essential for docking of 6-substituted 1-benzyl-3-(3,5-dimethylbenzyl)uracils. In addition, Y181C loses important aromatic ring interactions in the core of the NNRTI-binding pocket, decreasing binding of NNRTIs (42). Taken together, the results of sequence analysis and docking studies indicate that AmBBU requires the interaction(s) with Tyr181 for its inhibitory effect on HIV-1.

In addition to Y181C, we identified four mutations in the RNase H domain of RT: K451R, R461K, T468P, and D471N (Fig. 3). Since we do not know exactly when these mutations emerged in relation to Y181C, it is difficult to elucidate their precise role in acquisition of HIV-1 resistance to AzBBU and AmBBU. One possibility is that mutations in the RNase H domain are merely the result of polymorphisms of RT and are not related to Y181C, as indicated by several HIV-1 (subtype B) prototype strains containing some of the identified mutations in the RNase H domain (Table 3). The strains MCK1_LAI_III_B and PV22_LAI_III_B, for instance, display all four amino acid changes but are not associated with any common NNRTI-resistant mutation. However, some strains display one or more NNRTI-resistant mutations in association with the mutation T468P/S, suggesting that mutations in the RNase H domain could act in coordination with NNRTI-resistant mutations to favor drug resistance. It has been postulated that drug resistance mutations reduce the replication fitness of HIV-1 (10). NNRTI-resistant RTs with the Y181C mutation have been shown to alter the rate of one or both modes of RNase H cleavage with no significant effects on RNA- or DNA-dependent DNA polymerization, and a decrease in RNase H activity has been associated with greater reductions in replication fitness (2).

In 2005, Nikolenko et al. suggested that mutations in the RNase H domain could significantly contribute to an increase of RT resistance to NRTIs (37). Later, the same group proposed the RNase H-dependent NNRTI-resistant model, which suggests that mutations in the RNase H domain that reduce RNase H cleavage will allow more time for the NNRTIs to dissociate from the NNRTI-RT-template/primer complex, allowing the reinitiation of polymerization and thereby resulting in enhanced NNRTI resistance (36). Thus, combining mutations in RT that reduce NNRTI affinity with mutations that reduce RNase H cleavage should further increase NNRTI resistance (24, 36). In an RNase H-independent mechanism, NNRTIs themselves can increase RNase H activity, so that mutations reducing RNase H activity are selected in response to NNRTI therapy, because they restore the balance between RNase H activity and polymerization (16). An alternative explanation is that NNRTIs may inhibit HIV-1 replication by increasing RT dimer stability (51). Thus, NNRTI-binding pocket mutants that confer drug resistance should decrease the stability of RT heterodimers. In general, the nucleic acid structure-dependent interplay between polymerase and RNase H domains is likely to affect overall efficacy of NNRTIs against HIV-1 replication, as well as the selection of mutations in the NNRTI-binding site associated with NNRTI resistance.

The effects of mutations in the RNase H domain on NNRTI resistance have been confirmed *in vitro*, and yet their clinical impact is still unclear. Current HIV-1 genotypic analyses of patients generally focus on the N terminus of the polymerase domain, thus missing important information on mutations in the thumb-connection subdomains and RNase H domain that might be related to resistance either alone or in combination with other RT mutations. Yap et al. showed that N348I in the connection subdomain was highly prevalent in a patient cohort and was highly associated with thymidine analogue-associated mutations (TAMs) and the NNRTI mutations K103N and Y181C (59). Hachiya et al. also

examined N348I in treatment-experienced clinical isolates from Japan and found that N348I was prevalent in AZT and/or ddI therapy and that several mutations in the connection subdomain and RNase H domain typically acted as pretherapy polymorphisms (20, 21). Waters et al. found N348I prevalent in treatment-experienced patients (54). In addition, these researchers found that the genotypic profiles of patients with or without the K451R mutation within a treatment-experienced group showed a higher incidence of NNRTI mutations in patients with the K451R mutation. Santos et al. analyzed 450 sequences from Brazilian subtype B isolates and public databases and found nine mutations in the connection subdomain and six mutations in the RNase H domain that were associated with NRTI therapy (45). Positions K451 and D471 were less conserved in NRTI-experienced patients, while R461 and T468 were equally variable in both naive and experienced patients. A comparison of RNase H sequences in naive versus NRTI-experienced patients in a French cohort showed that mutations L469T/I/M/H, T470P/S/E/K, A554T/L/K, and K558R/G/E were more prevalent among treatment-experienced patients (44). However, Ntemgwa et al. analyzed RNase H mutations in NRTI-experienced patients from a Canadian and an Italian cohort and found positions D460, P468, H483, K512, and S519 to be extensively polymorphic in both naive and experienced patients (38). Recently, an analysis of patient sequences from databases showed that several mutations in the connection subdomain were significantly higher for sequences that contained one or more RTI resistance mutations compared to sequences without RTI resistance mutations (16). Moreover, subtype B-infected patient database analysis showed that RNase H mutations, including K451R, increased in frequency with the number of TAMs in a dose-dependent fashion (31). That study demonstrated that distinct RT C-terminal mutations can act as primary or secondary drug resistance mutations and are associated in a complex array of phenotypes with RT polymerase domain mutations.

In this *in vitro* study, we identified four mutations in the RNase H domain that might be related to the NNRTI resistance mutation Y181C. Biochemical studies are needed to understand the molecular mechanism of the associations and interactions between mutations within the polymerase and RNase H domains of RT. Further experiments are under consideration to validate the role of these mutations in the acquisition of resistance to AzBBU and AmBBU. First, viral strains containing the identified mutations in the RNase H domain, such as MCK1_LAI_IIIB and PV22_LAI_IIIB, will be used for testing the uracil derivatives, since these strains have all four amino acid changes but are not associated with any common NNRTI-resistant mutation. Second, recombinant RT enzymes containing the identified mutations will be used to determine the inhibitory effects of AzBBU and AmBBU on their catalytic activity. Third, an experiment on site-directed mutagenesis will be performed to elucidate the precise role of the RNase H mutations in the acquisition of resistance.

In terms of drug development, we found useful information for the future design of 6-substituted uracil derivatives with enhanced chemical properties that improve anti-HIV-1 activity and resistance profiles. Although the chemical properties of AzBBU and AmBBU suggest a good drug-likeness profile, further studies are required to assess the toxicity and pharmacokinetics of 6-substituted uracil derivatives *in vivo*. A limitation of our study relates to the use of laboratory strains. However, the mutations that we identified have been reported in clinical isolates (38, 45, 54). Another limitation of our study relates to the lack of information on the mutations in the RNase H domain of NNRTI-treated patients, since these mutations have been reported mostly in NRTI-treated patients. Furthermore, in a clinical setting, NNRTIs must be used in combination therapy, which may alter the pattern for resistance. Thus, the emergence of drug resistance should be further investigated and confirmed in clinical trials.

In conclusion, our results provide important information on the acquisition of resistance to the novel uracil derivatives AzBBU and AmBBU. Although the initial mutation Y181C can be sufficient in the acquisition of HIV-1 resistance, additional mutations in the RNase H domain of RT could additionally be associated to the mechanisms of resistance. Docking studies using the crystal structure of RT showed that AmBBU requires the amino acid residues Leu100, Val106, Tyr181, and Trp229 for its inhibitory effect on HIV-1. Further studies are necessary to determine the precise role of each mutation in the acquisition of HIV-1 resistance to the present compounds.

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Identification of Novel Inhibitors of Human Immunodeficiency Virus Type 1 Replication by *In Silico* Screening Targeting Cyclin T1/Tat Interaction

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Human immunodeficiency virus type 1 (HIV-1) transcription is essential for viral replication and the only step for viral genome amplification. Cyclin T1 (CycT1) interacts with HIV-1 Tat and transactivation-responsive (TAR) RNA, leading to the activation of viral transcription through the hyperphosphorylation of RNA polymerase II (RNAPII). Thus, the CycT1/Tat/TAR RNA interaction represents a novel target for inhibition of HIV-1 replication. In this study, we conducted *in silico* screening of compounds targeting the CycT1/Tat/TAR RNA complex and found that two structurally related compounds (C1 and C2) had high docking scores for a model of the complex. These compounds proved inhibitory to HIV-1 replication in tumor necrosis factor alpha-stimulated chronically infected cells. In addition, C3, a derivative of C1 and C2, was found to be a more potent inhibitor of HIV-1 replication in chronically infected cells. C3 also inhibited HIV-1 replication in acutely infected cells. The compound could suppress Tat-mediated HIV-1 long terminal repeat-driven gene expression and phosphorylation of RNAPII through inhibition of Tat binding to CycT1. Furthermore, the docking pose of C3 was defined by analyses for its *in silico* docking energy and *in vitro* antiviral activity, which indicates that C3 interacts with Tat-binding amino acids of CycT1. Thus, a series of compounds described herein are novel inhibitors of HIV-1 transcription through inhibition of CycT1/Tat interaction.

The current antiretroviral therapies (ART) against human immunodeficiency virus type 1 (HIV-1) have proved highly effective in reducing viral load and delaying disease progression in infected patients (1). However, even such effective therapies cannot completely eradicate the virus from their bodies, and treatment interruption generates a rebound of viral load from certain reservoir cells chronically infected with HIV-1 (2). Thus, infected patients need to be continuously treated with antiretroviral drugs for a long period, presumably throughout their life, which leads to serious concerns about the emergence of drug-resistant viruses and chronic adverse effects of the drugs. Considering the fact that effective vaccines against HIV-1 are not available (3), the development of novel antiretroviral drugs with a different mechanism of action is still mandatory.

Transcription of HIV-1 genome RNA from its proviral DNA is a crucial step in the viral life cycle, and the amplification of genetic information occurs only at this step. HIV-1 transcription is predominantly controlled at the step of RNA elongation by the virus-encoded transcriptional activator protein Tat (4–6). Tat directly binds to cyclin T1 (CycT1), a subunit of positive transcription elongation factor b (P-TEFb), which is composed of CycT1 and cyclin-dependent kinase 9 (CDK9) (7). Tat recruits P-TEFb to transactivation-responsive (TAR) RNA located at the 5′ end of nascent HIV-1 transcripts (6–10). Subsequently, the CDK9 subunit of P-TEFb phosphorylates Ser2 of the heptad repeats in the C-terminal domain (CTD) of RNA polymerase II (RNAPII), which is a marker of the transcriptional transition from initiation to elongation. The phosphorylated RNAPII starts the elongation of HIV-1 transcripts. Thus, the complex formation of P-TEFb/Tat/TAR RNA is essential for the amplification of HIV-1 genome RNA, and their interfaces are considered to be target sites for novel intervention in HIV-1 transcription.

Human CycT1 is comprised of 726 amino acids and contains a cyclin box repeat domain (amino acids 31 to 250), a coiled-coil sequence (amino acids 379 to 530), and a PEST sequence (amino acids 709 to 726) (7, 11). The N-terminal amino acids (amino acids 1 to 272) of CycT1 are sufficient to bind Tat and TAR RNA and to mediate transactivation by Tat (12). A previous study of mutant CycT1 demonstrated that the Tat/TAR RNA recognition motif (TRM) of CycT1 (amino acids 250 to 262) was essential for CycT1/Tat/TAR RNA complex formation. In particular, N250, R259, and C261 of the TRM were crucial for Tat binding, while R251, L252, R254, I255, and W258 were required for TAR RNA binding (13). Thus, the TRM region of CycT1 is a possible target of compounds for inhibition of HIV-1 transcription. In addition, two crystallographic structures of CycT1 with a viral factor(s) have recently been reported: the equine CycT1/equine infectious anemia virus (EIAV) Tat/TAR RNA complex (Protein Data Bank [PDB] identity [ID]: 2W2H) (14) and human CycT1/HIV-1 Tat complex (PDB ID: 3MIA) (15). The structures of these complexes provide the interactive information between CycT1 and a viral factor(s) in further detail. Although the structure of human P-TEFb/HIV-1 Tat reported by Tahirov et al. revealed the interface between CycT1 and Tat, the TRM could not be elucidated because of its distorted structure (15). On the other hand, Anand et al. demonstrated the structure of equine CycT1/EIAV Tat/TAR RNA, including the TRM region, in which the TRM of equine

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CycT1 interacted with EIAV Tat but not with EIAV TAR RNA in this complex (14). Thus, although the structure of human CycT1/HIV-1 Tat/TAR RNA has not fully been clarified yet, these two structure models provide useful information on possible targets of small-molecule compounds for intervention in HIV-1 transcription.

There are several reports on the identification of small-molecule inhibitors of HIV-1 transcription, and most of the inhibitors target CDK9 or TAR RNA (16–19). We have also reported two compounds, K-37 (20) and JTK-101 (21), as potent and selective inhibitors of HIV-1 transcription. As mentioned above, the interfaces between CycT1 and a viral factor(s) have recently been elucidated at the molecular level (14, 15). Therefore, it seems important to determine whether these interfaces can be used for identifying novel anti-HIV-1 agents. In this study, we conducted *in silico* screening of 3,000,000 compounds targeting the TRM of human CycT1 based on the structure of equine CycT1/EIAV Tat/TAR RNA and found that some compounds selected by the screening also inhibited HIV-1 replication *in vitro*. The most active compound proved to suppress Tat-mediated HIV-1 long terminal repeat (LTR)-driven gene expression and phosphorylation of RNAPII through inhibition of Tat binding to CycT1.

MATERIALS AND METHODS

Cells. OM-10.1 (22), U1 (23), CEM (21), and MOLT-4 (24) cells and peripheral blood mononuclear cells (PBMCs) were used in the anti-HIV-1 assays. OM-10.1 and U1 cells are clones of HL-60 and U937 cells latently infected with HIV-1, respectively. PBMCs were obtained from healthy donors and stimulated with phytohemagglutinin (PHA; Sigma-Aldrich, St. Louis, MO). W-3 and KM-3 cells were used for a reporter assay. W-3 and KM-3 cells are clones of CEM cells that stably integrate an HIV-1 LTR-driven secreted alkaline phosphatase (SEAP) gene (25). The integrated HIV-1 LTR contains two intact nuclear factor κB (NF-κB)-binding sites in W-3 cells, whereas both of the sites are mutated in KM-3 cells.

Compound database and conformer generation. A compound database containing approximately 3,000,000 molecules was obtained from Namiki, Tokyo, Japan. All *in silico* studies were performed using Molecular Operating Environment (MOE) software (Chemical Computing Group, Montreal, Canada). To select drug-like compounds, the database was filtered with the following conditions: molecular weight, 350 to 600; logP, 0 to 6; number of hydrogen bond donors or acceptors, <13; and number of rotatable bonds, <7. Partial charges were added to compounds, and at maximum 250 conformers per one compound were generated using the force field Merck Molecular Force Field 94x (MMFF94x) (26–28).

***In silico* screening of compounds.** Using MOE software, a model structure of human CycT1 was constructed by homology modeling based on the X-ray crystal structure of a complex composed of equine CycT1, EIAV Tat, and EIAV TAR RNA (PDB ID: 2W2H) (14), which were available from the PDB at the Research Collaboration for Structural Bioinformatics (http://www.rcsb.org/pdb/home/home.do). After addition of hydrogen atoms and assignment of atomic charges, the constructed human CycT1 model was subjected to energy minimization using the force field MMFF94x (26–28). Alpha Site Finder, a function of MOE, was used to search the target sites for *in silico* screening, where compounds could bind, in the human CycT1 model. We selected a pocket containing the TRM of CycT1 as a target site. MOE-ASEDock 2005 (Ryoka Systems, Tokyo, Japan) (29) was used for docking drug-like compounds to the target site. The docking state was evaluated by the docking energy calculated by MOE software, and compounds with high docking energy scores were selected and purchased from Namiki for further evaluation *in vitro*.

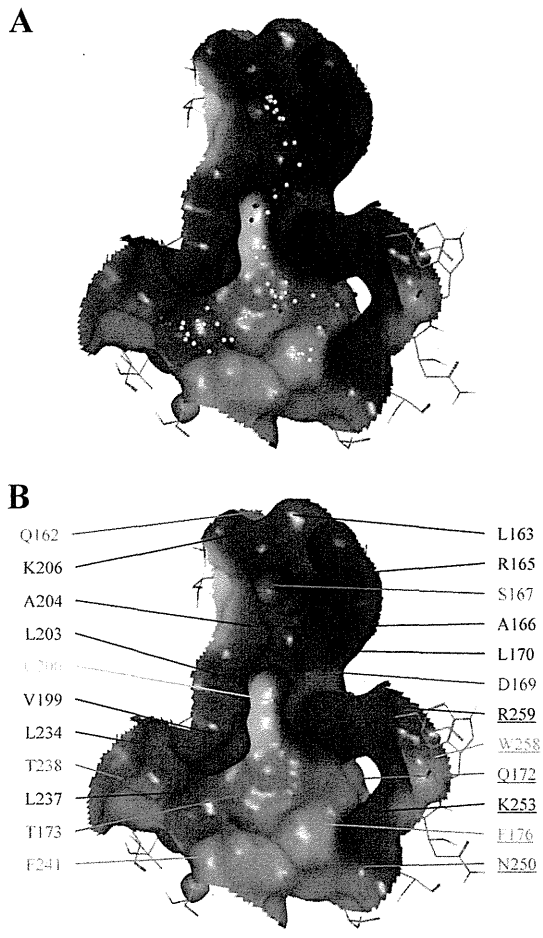

FIG 1 Molecular model for *in silico* screening. The molecular surface of the target site in human CycT1 model is shown. (A) Target site for *in silico* screening determined by MOE software. The white and red spheres indicate the hydrophobic and hydrophilic properties, respectively. These spheres were used for docking of compounds. (B) Amino acids of CycT1 within 4.5 Å from the spheres in panel A. The amino acids interacting with Tat or TAR RNA are underlined. The target sites are shown in color according to the amino acid properties. Red (D), blue (R and K), light green (F and W), dark green (A, L, and V), and magenta (T, Q, N, and S) indicate acidic, basic, aromatic, and neutral hydrophilic amino acids, respectively.

Anti-HIV-1 assays. The anti-HIV-1 activity of test compounds in chronically infected cells was based on the inhibition of HIV-1 p24 antigen production in OM-10.1 and U1 cells stimulated with tumor necrosis factor alpha (TNF-α) (Roche Diagnostics, Mannheim, Germany). Briefly, OM-10.1 and U1 cells (1×10^5 cells/ml) were incubated in the presence of various concentrations of the compounds for 24 h and stimulated with 0.1 ng/ml of TNF-α. After incubation for 3 days at 37°C, the culture supernatants were collected, and their p24 antigen levels were determined with a sandwich enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrix, Buffalo, NY). The anti-HIV-1 activity of the compounds in acutely infected cells was based on the inhibition of p24 antigen production in CEM cells, MOLT-4 cells, and PBMCs infected with HIV-1 (HTLV-III_B strain). CEM and MOLT-4 cells were infected with HIV-1 at a multiplicity of infection (MOI) of 0.001, while PBMCs were infected with HIV-1 at an MOI of 0.01. After viral adsorption for 2 h, the cells were washed thoroughly with culture medium to remove unabsorbed viral particles. The infected cells (1×10^5 cells/ml) were cultured in the presence of various concentrations of the compounds. After incubation for 3 days at 37°C, the cells were subcul-