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High-Level Resistance to (−) Enantiomeric 2′-Deoxy-3′-Thiacytidine In Vitro Is Due to One Amino Acid Substitution in the Catalytic Site of Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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Passage of human immunodeficiency virus type 1 in the presence of increasing 2′-deoxy-3′-thiacytidine (3TC) concentrations results in high-level (>100-fold) 3TC-resistant viruses. All 3TC-resistant viruses possess a substitution at the second codon (from a methionine into an isoleucine) at position 184 within the highly conserved motif (YMDD) of human immunodeficiency virus type 1 reverse transcriptase. 3TC-resistant viruses were cross-resistant to the (−) enantiomer of the fluorinated derivative of BCH-189 but remained susceptible to 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine. The susceptibilities of the 3TC-resistant viruses to the (+) enantiomers of BCH-189 and the fluorinated derivative of BCH-189 demonstrate an enantiomeric specificity for viruses selected under these conditions. Introduction of an isoleucine substitution at codon 184 into a background of two known 3′-azido-3′-deoxythymidine resistance mutations (amino acids 41 and 215) restored the susceptibility of this virus to 3′-azido-3′-deoxythymidine.

The nucleoside analogs 2′,3′-dideoxyinosine (ddI) and 2′,3′-dideoxycytosine (ddC) have been shown to inhibit human immunodeficiency virus (HIV) replication in vitro and in vivo (3, 24, 25). These 2′,3′-dideoxynucleosides (ddI and ddC) lack the hydroxyl group at the 3′ carbon of the sugar group. Another nucleoside analog, 3′-azido-3′-deoxythymidine (AZT), with in vivo activity contains a 3′-azido group instead of the 3′-OH group. Recently, a novel nucleoside analog, 2′-deoxy-3′-thiacytidine, in which the 3′ carbon of the ribose in ddC is replaced by a sulfur atom, was described (20). The racemic mixture of 2′-deoxy-3′-thiacytidine (BCH-189) has potent in vitro anti-HIV type 1 (anti-HIV-1) activity (6). The (+) and (−) enantiomers of 2′-deoxy-3′-thiacytidine are equipotent in their antiviral activities, but the (−) enantiomer (3TC) is considerably less cytotoxic than the (+) enantiomer (5). Clinical trials are in progress to determine the efficacy and toxicity of 3TC.

The absence of a free 3′-OH group accounts for the major antiretroviral effect of these nucleoside analogs; once incorporated into the growing chain, no further nucleosides can be added (chain termination).

The nature of mutations in the *pol* gene generated by in vitro selection of drug-resistant isolates with either zidovudine or the nonnucleoside reverse transcriptase (RT) inhibitors is to a large extent predictive of the mutation patterns observed in patients during treatment (2, 11, 14). Therefore, we investigated the appearance of 3TC-resistant isolates in vitro. Two protocols were used to generate 3TC-resistant isolates. Two established HIV-1 laboratory strains (HIV-1 HXB2, HIV-1 RF) as well as one primary isolate were passaged in different host cells derived from continuous T-cell lines (MT-2 and C8166). All preselection isolates were susceptible to ddI, ddC, and AZT. During continuous passage of these viruses, increasing concentrations of 3TC were used to select for resistant variants.

Both approaches were successful in generating 3TC-resistant isolates. Nucleoside sequence determination of the RT gene from all three 3TC-resistant strains revealed an amino acid change at codon 184. The biological significance of this change was confirmed by generation of recombinant viruses and subsequent susceptibility testing.

Cells and viruses. C8166 cells (18), MT-2 cells, and MT-4 cells (10) were routinely grown at 37°C in 5% CO_2 in RPMI 1640 growth medium (Flow Laboratories) containing 10% fetal calf serum, 2 mM L-glutamine, 100 IU of penicillin per ml, and 10 μg of streptomycin per ml. HeLa cell line HT4-6C (4) was routinely grown in Dulbecco's modification of Eagle's medium containing 5% fetal calf serum and the antibiotics mentioned above.

Clinical isolate C19 was obtained from an HIV-1-infected subject who had not received antiretroviral therapy; the clinical isolate was obtained prior to the initiation of 3TC antiviral chemotherapy. The HIV-1 RF strain was obtained from the AIDS Reagent Project of the Medical Research Council of the United Kingdom (South Mimms, United Kingdom).

Selection of 3TC-resistant viruses. (i) Protocol A. C8166 cells (10^6 cells per ml) were infected with the HIV-1 RF strain at a multiplicity of infection of 1 infectious dose per cell. 3TC was initially added to the culture at 0.003 μM. The culture was incubated until a cytopathic effect was detectable (3 to 4 days). Supernatants were titrated in fresh C8166 cells at a double concentration of 3TC. The culture with the highest dilution of virus showing a cytopathic effect was harvested and passaged further with an increased 3TC concentration as described above. After approximately 10

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TABLE 1. Drug susceptibility profiles of HIV-1 isolates selected in vitro

Isolate	Status	Selection protocol	IC_{50} (μM)[a]							Codon at position 184
			3TC	(−)-FTC	AZT	ddI	(+)-BCH-189	(+)-FTC	ddC	
HIV-1 RF	Wild-type	A	0.16	2.02	0.056	7.63	3.50	2.10	0.22	Met
	Selected	A	>437	323	0.015	12.3	3.71	7.07	0.22	Ile
HXB2	Wild-type	B	0.39	0.105	0.02	3.18	0.91	6.85	0.42	Met
	Selected	B	>437	9.29	0.001	3.82	0.57	1.7	0.4	Ile
C19[b]	Wild-type	B	1.25	ND[c]	ND	ND	ND	ND	ND	Met
	Selected	B	92	ND	ND	ND	ND	ND	ND	Ile
HIV-1 IIIB	Wild-type	C[d]	0.8	ND	0.04	19	ND	ND	0.45	Met
	Selected	C	936	ND	0.05	76	ND	ND	2.5	Val

[a] The results represent the averages of at least two experiments performed in duplicate. The error was <15%. The correlation coefficients for the data were >0.95.
[b] HIV-1 clinical isolate.
[c] ND, not done.
[d] HIV-1 3TC-resistant variants were selected in MT-4 cells (data from reference 8).

passages, 3TC-resistant virus stock was prepared by passaging three times in 457 μM 3TC.

(ii) Protocol B. The selection procedure for protocol B has been described previously (14). In brief, MT-2 cells were infected with cell-free virus (either HXB2 or clinical isolate C19) in the presence of 0.2 μM 3TC. The culture was monitored for syncitium formation. Once syncitia were detectable throughout the culture, the cell-free supernatant was used to inoculate fresh MT-2 cells in the presence of a double concentration of 3TC. After nine passages, a virus stock was harvested for susceptibility testing.

Drug susceptibility assays. (i) Protocol A. Aliquots of MT-4 cells (10^6 cells per ml) in RPMI 1640 growth medium were infected with wild-type HIV-1 RF strain or 3TC-resistant virus at a multiplicity of infection of 10^{-3} infectious doses per cell. Infected or mock-infected cell suspensions were inoculated into 96-well microtiter plates containing serial 10-fold dilutions of test compounds (final concentration, 5×10^4 cells per well). Experiments were incubated for 7 days at 37°C in 5% CO_2 and developed with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] by the method of Pauwels et al. (16).

(ii) Protocol B. The drug susceptibility procedure for protocol B has been described previously (13) and was a plaque reduction assay in HeLa CD4 cells. In short, the inhibition by drug of the formation of plaques (foci of multinucleated giant cells) was determined by infecting monolayers of HeLa CD4 *lacZ* cells with cell-free virus. 3TC was serially diluted from 100 to 0.02 μM in these assays.

Two protocols, protocols A and B, were successful in generating 3TC-resistant isolates (Table 1). The fold increase in the 50% inhibitory concentrations (IC_{50}s) of 3TC, as measured in several assays, ranged between 100- to 1,000-fold. Both 3TC-resistant laboratory isolates retained the susceptibilities to AZT, ddC, and ddI. Previously published comparative data for 3TC-resistant HIV-1 IIIB derived by passaging in MT-4 cells (protocol C) showed a four- to fivefold reduced susceptibility to ddC and ddI (8). Furthermore, 3TC-resistant viruses were resistant to the (−) enantiomer of the fluorinated derivative of BCH-189 [(−)-FTC] but remained susceptible to the (+) enantiomer of BCH-189 and to (+)-FTC.

Genetic analysis of in vitro selected 3TC-resistant viruses. In order to determine the genetic changes that occur during passage of HIV-1 in the presence of 3TC, the nucleotide sequence of the RT gene was determined for all three 3TC-resistant strains.

The RT fragment from MT-2 cells containing HIV-1 HXB2 or HIV-1 RF was amplified by polymerase chain reaction (PCR). The first PCR primer pair was located in the integrase and protease regions (5′-GGA AAC CAA AAA TGA TAG GGG GAA TTG GAG G-3′ and 5′-TCT ACT TGT CCA TGC ATG GCT TC-3′, respectively). The 5′ primer of the second PCR primer pair consisted of 1:1 mixture of 5′-TTG CAC TTT GAA TTC TCC CAT TAG-3′ AND 5′-TG TAC TTT GAA TTC CCC CAT TAG-3′. The 3′ primer comprised 5′-CTT ATC TAT TCC ATC TAG AAA TAG T-3′. DNA was purified by GeneClean (Stratech, London, United Kingdom) and was ligated into a modified pSP73 plasmid (Omega) and transformed in the *Escherichia coli* C600. DNA was obtained from individual bacterial clones and purified. Nucleotide sequence analysis of RT sequences was performed as described by Schuurman and Keulen (18). After digestion with restriction enzymes, the clonal RT fragment was cotransfected with a HXB2-D molecular clone that lacked the RT coding region (pHIV Δ RT) into SupT1 cells as described previously (11). Cell cultures were monitored for the development of syncitia. Cell-free virus was obtained from culture supernatants and titrated, and drug susceptibility was tested in the HeLa-CD4 plaque assay. The RT from the resistant HXB2 isolate showed only one amino acid change compared with the respective preselection strain from a methionine to an isoleucine at codon 184 (Table 1).

The complete RT gene of one clone obtained from the 3TC-resistant HIV-1 RF contained the isoleucine codon at position 184 and two other coding changes (Ile to Val at position 505 and Glu to Gly at position 529).

The sequence of C19 was analyzed by direct sequencing by the procedure described above (18). The change at the isoleucine (ATA) codon at position 184 was the only change from the wild-type sequence, suggesting that the isoleucine codon is present in the majority of amplified RT genes.

Generation of recombinant viruses with Ile at codon 184. The biological significance of the amino acid change at position 184 was confirmed by generating recombinant viruses containing the isoleucine codon and subsequent testing of their susceptibilities.

Mutant RT genes encoding an isoleucine (ATA) instead of

TABLE 2. Susceptibilities of recombinant viruses to 3TC

Recombinant virus[a]	Amino acid at position[b]:			IC_{50} (μM)	
	41	215	184	3TC	AZT
HXB2 (F1-3)	wt	wt	wt	0.6	ND[c]
HXB2 (F1-3/184I)	wt	wt	M	>100	ND
H257-6	M	M	wt	4.4	2.8
H257-6/184I	M	M	M	>100	0.05

[a] Recombinant viruses are described in the text.
[b] wt, wild-type amino acids are M41, T215, and M184; M, mutant amino acids are 41L, 215Y, and 184I.
[c] ND, not done.

a methionine (ATG) at amino acid position 184 of HXB2-RT were generated by site-directed mutagenesis. pF1-3 (pSP73 containing the complete RT gene of HXB2) was used as a template for mutagenesis by PCR. The PCR contained two oligonucleotide primers, one of which overlapped the codon at position 184 and encoded the ATA mutation. Moreover, this oligonucleotide encoded an *Xho*II restriction site at its 5′ end. At a distance of 113 bp from the *Xho*II site, the amplified fragment encoded a *Bst*XI site. After amplification, the fragment was digested with *Xho*II and *Bst*XI, thereby creating a 113-bp fragment containing the required mutation. This fragment was used to exchange the wild-type codon at position 184, which was present in pF1-3, with the isoleucine mutant, thereby creating pF1-3/184I. The nucleotide sequence of the complete 130-bp *Xho*II-*Bst*XI fragment present in pF1-3/184I was verified by sequence analysis. Similarly, the isoleucine mutation at position 184 was introduced into the RT gene obtained from an AZT-resistant HIV-1 isolate (H257-6) derived from an AZT-treated individual. After transfection, genomic DNA was isolated from the recombinant virus. The presence of the two known AZT mutations (at positions 41 and 215) and the 3TC mutations (at position 184) were confirmed by DNA sequence analysis (H257-6/184I).

For recombinant viruses with an isoleucine at position 184, there was a more than 100-fold increase in the IC_{50} of 3TC (Table 2). An interesting phenomenon was observed when the Met to Ile mutation at position 184 was introduced into the clinical HIV-1 isolate containing a background of two AZT resistance-conferring mutations at codons 41 (leucine) and 215 (tyrosine). The resulting virus (H257-6/184I) was resistant to 3TC, whereas AZT resistance declined approximately 50-fold in comparison with that for the parental recombinant virus, H257-6. Two different protocols of tissue culture passage under drug pressure were used to generate HIV variants that were resistant to 3TC. Cloning and sequencing studies revealed that an amino acid substitution at codon 184 was responsible for the resistance phenotype. Site-directed mutagenesis procedures have confirmed that a single amino acid change from methionine to isoleucine at codon 184 can confer high-level resistance to 3TC. It is interesting that codon 184 is situated in a highly conserved region of the viral reverse transcriptase; amino acids 183 to 186 are reported to be essential for correct enzyme function, and the second amino acid in this motif is a methionine in HIV-1, HIV-2, human T-cell lymphotropic virus types I and II, and Rous sarcoma virus (1). The methionine in the YMDD motif has a critical role in the activity of HIV-1 RT and the subsequent production of infectious virus (15). Substitution of tyrosine, leucine, glycine, or proline at codon 184 reduced enzyme activity by 80% or more. However, the in vitro activity of RTs with the methionine replaced by a valine were 70 to 100% as active as the RT from the wild-type strain, and proviruses containing this substitution produced viruses with infectivities similar to that of the wild type (23). A valine substitution at codon 184 has previously been identified by passage of HIV under ddI drug pressure. That HIV isolate also demonstrated reduced susceptibilities to ddC, 3TC, and racemic 2′-deoxy-3′-thiacytidine (BCH-189) (8, 9). In addition, viruses selected under ddC or BCH-189 drug pressure showed both reciprocal cross-resistance and resistance to ddI but not AZT (7). An isoleucine substitution at codon 184 conferred much greater specificity, and these viruses retained their susceptibilities to ddC and ddI. All 3TC-resistant viruses were susceptible to the (+) enantiomers of BCH-189 and FTC. Further work is required to determine the mechanism of this enantiomeric specificity. A recent report describing serial passage of HIV-1 in 3TC or (−)-FTC revealed valine and isoleucine substitutions at codon 184, but enantiomeric specificity was not demonstrated in drug susceptibility assays with the (+) enantiomer of BCH-189 and (+)-FTC (17).

The lack of cross-resistance between 3TC-resistant viruses and AZT supports the possible use of combination therapy to treat HIV-infected individuals. Synergistic activity has been demonstrated between 3TC and AZT (22), and their clinical use together may serve to stall the development of viral resistance. In this regard, it was interesting that an isoleucine substitution at codon 184 in a background of the known AZT resistance mutations at codons 41 and 215 conferred resistance to 3TC but restored susceptibility to AZT. Similar observations have been made with a substitution at codon 74 (which is known to confer ddI resistance) and codon 181 (which is known to confer nevirapine resistance) in a background of AZT mutations (12, 21).

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