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Characterization of Human Immunodeficiency Viruses Resistant to Oxathiolane-Cytosine Nucleosides

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The (−) enantiomers of 2′,3′-dideoxy-5-fluoro-3′-thiacytidine [(−)-FTC] and 2′,3′-dideoxy-3′-thiacytidine [(−)-BCH-189] were recently shown to inhibit selectively human immunodeficiency viruses (HIV) and hepatitis B virus in vitro. In the current study, the potential for HIV type 1 (HIV-1) resistance to these compounds was evaluated by serial passage of the virus in human peripheral blood mononuclear cells and MT-2 cells in the presence of increasing drug concentrations. Highly drug-resistant HIV-1 variants dominated the replicating virus population after two or more cycles of infection. The resistant variants were cross-resistant to (−)-FTC, (−)-BCH-189, and their (+) congeners but remained susceptible to 2′,3′-dideoxycytidine, 3′-azido-3′-deoxythymidine, 3′-fluoro-3′-deoxythymidine, 2′,3′-dideoxyinosine, phosphonoformate, the TIBO compound R82150, and the bis(heteroaryl)piperazine derivative U-87201E. Reverse transcriptase derived from drug-resistant viral particles was 15- to 50-fold less susceptible to the 5′-triphosphates of FTC and BCH-189 compared with enzyme from parental drug-susceptible virus. DNA sequence analysis of the reverse transcriptase gene amplified from resistant viruses consistently identified mutations at codon 184 from Met (ATG) to Val (GTG or GTA) or Ile (ATA). Sequence analysis of amplified reverse transcriptase from a patient who had received (−)-BCH-189 therapy for 4 months demonstrated a mixture of the Met-184-to-Val (GTG) mutation and the parental genotype, indicating that the Met-184 mutation can occur in vivo. The Met-184 residue lies in a highly conserved polymerase motif (Tyr-Met-Asp-Asp) adjacent to the putative catalytic site of the HIV-1 reverse transcriptase composed of the carboxylate triad Asp-110, Asp-185, and Asp-186. Substitution of the Met-184 residue appears to markedly affect the anti-HIV activity of oxathiolane-cytosine analogs.

The (−) enantiomers of 2′,3′-dideoxy-5-fluoro-3′-thiacytidine [(−)-FTC] and 2′,3′-dideoxy-3′-thiacytidine [(−)-BCH-189 or 3TC], nucleoside analogs with the unusual L configuration, were recently shown to have potent antiviral activity against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) and hepatitis B virus in cell culture (2, 5, 8, 9, 12, 15, 33, 35, 36). These antiviral agents are not cytotoxic in vitro and have a favorable pharmacological profile in animal models and in humans (32, 41). Additional preclinical studies have demonstrated that the 5′-triphosphates of (−)-FTC and (−)-BCH-189 are competitive inhibitors of HIV-1 reverse transcriptase (RT) and are potent DNA chain terminators when HIV-1 RT is used (13, 35). (−)-BCH-189 has progressed to phase I clinical trials, and studies with humans with (−)-FTC will be initiated soon. The outcome of these studies will determine the ultimate value of these compounds for the prevention and treatment of HIV infection.

A growing concern in the evaluation of new antiretroviral agents is the possibility that drug-resistant HIV-1 variants will emerge in patients on therapy and limit clinical efficacy (26). Several reports have documented the development of HIV-1 resistance to 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxyinosine (DDI), and 2′,3′-dideoxycytidine (DDC) in patients on prolonged therapy with these nucleoside analogs (27, 38). More recently, the development of HIV-1 resistance to nonnucleoside RT inhibitors has been observed both in vitro and in vivo (20, 21, 23, 27, 29). Point mutations in the coding sequence of HIV-1 RT that confer the resistance to nucleosides and nonnucleoside inhibitors have been identified (26). In several instances, characterization of drug-resistant viral variants that arise in cell culture with drug selection has helped to predict the likelihood of resistance and the types of resistant variants that develop in treated individuals (12, 18, 20, 21, 23, 27). Since little information on HIV-1 resistance to oxathiolane-cytosine analogs is available, the studies presented in this report were undertaken to isolate and characterize HIV variants that are resistant to (−)-FTC and (−)-BCH-189.

MATERIALS AND METHODS

Chemicals. (−)-FTC, (−)-BCH-189, and their (+) congeners were synthesized in our laboratories as described previously (2, 6, 14). The TIBO analog R82150 [(+)-(5*S*)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1H)-thione] (25) was obtained from K. Parker (Brown University, Providence, R.I.). The bis(heteroaryl)piperazine derivative U-87201E nonnucleoside RT inhibitor (28) was obtained from the National Institutes of Health courtesy of G. Tarpley of the Upjohn Co. 3′-Fluoro-3′-deoxythymidine (FLT) was obtained from the National Institutes of Health. Phosphonoformic acid

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(PFA) was obtained from Astra Läkemedel AB, Södertälje, Sweden. 3-[4,5-Diamethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Company (St. Louis, Mo.). All other chemicals, analytical grade, were obtained from Sigma Chemical Company. Stock solutions (10 to 40 mM) of the antiviral compounds were prepared in sterile dimethyl sulfoxide and diluted to the desired concentration.

Cells and viruses. Human peripheral blood mononuclear (PBM) cells from healthy donors, seronegative for HIV-1 and hepatitis B virus, were isolated by a single-step Ficoll-Hypaque discontinuous gradient centrifugation (Sigma) and propagated as described previously (37). The prototype strain of HIV-1_{LAI} obtained from the Centers for Disease Control and Prevention, Atlanta, Ga., was used as the standard virus for the studies with human PBM cells.

H9 cells, kindly provided by R. C. Gallo (National Cancer Institute, Bethesda, Md.), and MT-2 cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases; contributed by D. Richman) were cultured in RPMI 1640 (Whittaker M. A. Bioproducts, Walkersville, Md.) with penicillin (50 IU/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (10 mM), and 10% dialyzed fetal bovine serum (JRH Biosciences, Lenexa, Kans.). Stock preparations of HIV-1 (HTLV-IIIB strain; R. C. Gallo) were harvested from infected H9 cells by the shakeoff method (20). Stock virus infectivity was determined by end-point dilution in PBM cells (HIV-1_{LAI}) or in MT-2 cells (HIV-1_{IIIB}) as described previously (18, 38).

Selection of drug-resistant viruses. (i) In PBM cells. Bulk HIV-1-infected 3-day-old phytohemagglutinin-stimulated human PBM cells (10^6 cells per ml) were placed in a 25-cm^2 flask and exposed to (−)-FTC and (−)-BCH-189 at a final concentration of 0.1 μM, which is approximately 30-fold the median effective antiviral concentration (EC_{50}) of these drugs against low-passage parental virus (33, 35). Untreated infected and uninfected cells served as controls for each passage. The cells were incubated with virus at a multiplicity of infection of 0.01 for 1 h before addition of the drug. After 1 week of incubation in 5% CO_2–air at 37°C, all the cells and media were removed from the flask and centrifuged at 700 × *g* for 5 min. The supernatant (3 ml) was saved for RT activity determination and for preparation of a virus stock for drug susceptibility assays. The RT activity of the supernatant obtained from virus-infected drug-treated cells was compared with that from supernatant obtained from infected untreated cells at different weekly cycles, as described previously (37). Half the cells and media were placed in a new flask containing uninfected mitogen-stimulated PBM cells, and fresh drug at the desired concentration was added. This procedure was repeated each week for a total of 8 weeks, except that at week 4, the (−)-FTC and (−)-BCH-189 concentrations were increased to 1 and 10 μM, respectively, and at week 6, the (−)-FTC concentration was increased to 10 μM. Virus stock from each weekly cycle was prepared by cell-free virus infection of human PBM cells, as described previously (37).

(ii) In MT-2 cells. Selection of resistant virus in MT-2 cells was performed as previously described (20). Briefly, 5 × 10^6 target cells were incubated for 2 h with 10^6 50% tissue culture infective doses of HIV-1_{IIIB} in the presence of (−)-FTC at an initial concentration of 5 μM. The cells were washed twice after infection and cultured in medium containing 5 μM (−)-FTC. After 4 days, cell-free viral progeny were harvested and used to initiate a new cycle of infection in the presence of drug. Breakthrough virus was evaluated for resistance after each passage by determining viral infectivity in MT-2 cells (end-point dilution) in the presence and absence of 5 μM (−)-FTC. HIV-1 resistant to 5 μM FTC dominated the replicating virus population after two cycles of infection. This resistant virus was then passaged sequentially at increasing (−)-FTC concentrations to a maximum of 50 μM.

Drug susceptibility determination. For virus derived from human PBM cells, drug susceptibility assays were performed in PBM cells with a multiplicity of infection of 0.01, as described previously (37). For virus propagated in MT-2 cells, drug susceptibility assays were performed in MT-2 cells as published previously, with modification (17, 20). Briefly, triplicate wells of 96-well plates containing 10^4 MT-2 cells per well were inoculated with HIV-1 at a multiplicity of 0.01 50% tissue culture infective doses per cell. Serial threefold dilutions of drug were added immediately after infection, and cell viability was quantitated after 7 days by the MTT-dye reduction method (17). The percentage of protection of cell viability was calculated with the formula $[(a - b/c - b) \times 100]$, in which a was the A_{550} of drug-treated virus-infected wells, b was the A_{550} of untreated infected wells, and c was the A_{550} of untreated uninfected wells.

PCR amplification and cloning of RT. The full-length coding sequence of HIV-1 RT was polymerase chain reaction (PCR) amplified from infected cells as published previously (18) with modifications. Briefly, amplifications were performed with 1 μg of purified DNA with the primer pair RT-MT1 5′ GATAAGCTTGGGCCTTATCTATTCCATC 3′ and RT-MT2 5′ GTAGAATTCTGTTGACTCAGATTGGT TGCAC 3′, which flanks the RT region of *pol* and includes small fragments of protease and integrase in the 1.74-kb amplimer. PCR amplifications were performed in a 100-μl mixture of 50 mM KCl–10 mM Tris-HCl (pH 8.3)–1.5 mM $MgCl_2$–0.001% (wt/vol) gelatin–5.0 U Amplitaq DNA polymerase–0.2 mM dGTP–0.2 mM dATP–0.2 mM dTTP–0.2 mM dCTP–30 pmol of each primer. PCR reagents were obtained from Perkin-Elmer Cetus (Norwalk, Conn.). Reaction mixtures were overlaid with 2 drops of mineral oil, heated to 95°C for 5 min, and subjected to 35 amplification cycles each consisting of 1 min at 95°C for denaturation, 1 min at 58°C for annealing, and 2 min at 72°C for extension. Samples were electrophoresed on a 0.8% agarose gel stained with ethidium bromide to determine product size and approximate yield. The 1.7-kb PCR product was sequenced directly (see below) or purified from agarose gels (Geneclean II kit, Bio 101, Inc., La Jolla, Calif.), ligated into the pCRII TA cloning vector (Invitrogen, San Diego, Calif.), and transfected into *Escherichia coli* INVαF'. Transformants were screened for the 1.7-kb insert by digestion with *Eco*RI. Plasmid DNA from appropriate clones was purified (Qiagen, Inc., Chatsworth, Calif.) and used as a template for sequencing. Sequencing was performed with the Promega fmol sequencing kit no. 04100 (Madison, Wis.) or the Sequenase kit no. 70770 (United States Biochemical, Cleveland, Ohio) according to the manufacturers' instructions.

Biochemical studies. Virus pellets were obtained from cell culture supernatants by high-speed centrifugation. The viral RT was obtained by disrupting the virus pellet as described previously (34). RT assays were performed with a reaction mixture containing 100 mM Tris HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 0.05 U of poly$(I)_n \cdot$ oligo$(dC)_{12-18}$ template-primer per ml, and 1 μM [^{3}H]dCTP (specific activity, 28.5 Ci/mmol), as described previously (35). Bovine serum albumin at a final concentration of 100 μg/ml was used in the RT

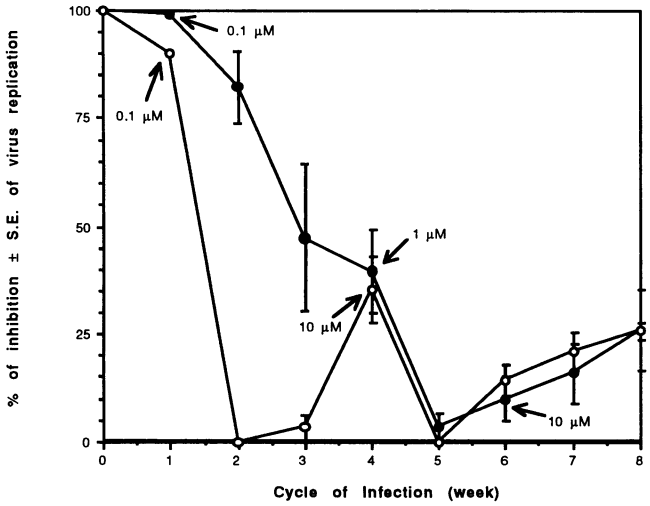

FIG. 1. HIV-1 breakthrough in the presence of (−)-FTC (●) and (−)-BCH-189 (○) in infected human PBM cells. Arrows indicate concentration of (−)-FTC or (−)-BCH-189 used for two or more weekly cycle periods to generate drug-resistant virus. Values are the means of sextuple replicates.

assay mixture to stabilize the viral enzyme. Racemic FTC-5′-triphosphate (FTC-TP), racemic BCH-189-5′-triphosphate (BCH-189-TP), and 2′,3′-dideoxycytidine-5′-triphosphate (DDC-TP) were used as potential inhibitors (35). Studies were also performed with recombinant Moloney murine leukemia virus RT obtained from Boehringer Mannheim Corp. (Indianapolis, Ind.). This enzyme was diluted 1:400 in water, and then 10 μl was added to each reaction mix. Other reaction conditions were the same as that used for HIV-1 RT except that $MnCl_2$ instead of $MgCl_2$ was used.

Patient sample. The patient was referred to our virology laboratory by a physician in private practice. This individual had been on AZT therapy for 2 weeks prior to treatment at the National Institutes of Health with (−)-BCH-189 (12 mg of drug per kg of body weight per day) in March 1992. His baseline $CD4^+$ count (170 cells per μl) increased almost threefold (450 cells per μl) after 2 months of (−)-BCH-189 therapy but declined to pretreatment levels (225 cells per μl) after 4 months, at which time drug therapy was discontinued. One month later, PBM cells were isolated by Ficoll-Hypaque centrifugation from a blood sample, the cells were cocultivated with fresh uninfected PBM cells for 10 days, and then the cellular DNA was purified for PCR amplification and RT sequence analysis.

Data analysis. The EC_{50} and median inhibitory concentrations were determined by the median effect method as described previously (7, 32).

RESULTS

Antiviral studies. To isolate HIV-1 variants with altered susceptibility to (−)-BCH-189 or (−)-FTC, HIV-1_{LAI} was passaged weekly in PBM cells in the presence of increasing concentrations of each drug (0.1 μM initially). Figure 1 demonstrates that the inhibition of HIV-1_{LAI} replication in PBM cells by (−)-BCH-189 and (−)-FTC decreased progressively with weekly passage despite increasing the drug concentrations. Breakthrough viral replication in the presence of 0.1 μM (−)-BCH-189 developed more rapidly (week 2) and was more complete than in that of 0.1 μM (−)-FTC (week 3 to 4). By the fifth week, however, viral replication in the presence of either 10 μM (−)-BCH-189 or 1 μM (−)-FTC equaled that of the untreated, infected cultures. Assays of viruses derived from weeks 2, 4, and 7 demonstrated that despite earlier viral breakthrough at week 2 a clear decrease in viral susceptibility to (−)-BCH-189 or (−)-FTC was not apparent until week 4 (data not shown). The drug susceptibility of parental HIV-1_{LAI} passaged in parallel in the absence of drug did not change by more than fivefold, which is within the experimental variability. Table 1 shows that week 7 viruses had become more than 1,000-fold resistant to both (−)-BCH-189 and (−)-FTC. Resistance to both compounds developed regardless of which had been used for initial selection, indicating cross-resistance between the compounds. Cross-resistance to the (+) enantiomers of BCH-189 and FTC was also evident (Table 1), but cross-resistance to DDC, DDI, AZT, FLT, TIBO R82150, or U-87201E was not present.

Resistant variants were also isolated by serial passage of virus in MT-2 cells in the presence of increasing concentrations of (−)-FTC (5 μM initially). Susceptibility testing demonstrated that after passage in 30 μM (−)-FTC, the virus was highly resistant to (−)-FTC (>300-fold) and cross-resistant to (−)-BCH-189 (>50-fold) but remained suscepti-

TABLE 1. Susceptibility of (−)-FTC- and (−)-BCH-189-resistant HIV-1 to oxathiolane-cytosine nucleosides and other antiretroviral agents in human PBM cells

Compound	Virus strain (EC_{50} [μM])[a]:			Increase in EC_{50} (fold)[b]	
	Parental HIV-1_{LAI}	(−)-FTC-resistant	(−)-BCH-189-resistant	(−)-FTC	(−)-BCH-189
(−)-FTC	0.03	51.1	49.2	1,703	1,640
(−)-BCH-189	0.07	>100	>100	>1,429	>1,429
(+)-FTC	0.84	92.4	29.3	110	35
(+)-BCH-189	0.21	>100	>100	476	476
DDC	0.14	0.03	0.89	0.2	6.4
DDI	1.2	4.6	0.84	3.8	0.7
AZT	0.004	0.02	0.003	5	0.8
FLT	0.08	0.05	0.05	0.6	0.6
TIBO R82150	0.09	0.18	0.36	2.0	4.0
U-87201E	1.3	2.4	3.2	1.8	2.5

[a] Virus stocks were obtained after seven or eight passages with or without drug. The results represent the averages of at least two experiments performed in duplicate. In general, the error was less than 10%. The correlation coefficients for the data were ≥0.95.

[b] Relative to parental strain passaged the same number of times.

TABLE 2. Susceptibility of (−)-FTC-resistant HIV-1 to oxathiolane-cytosine nucleosides and other antiretroviral agents in MT-2 cells

Compound	Virus strain (EC_{50} [μM])[a]:		Increase in EC_{50} (fold)
	Parental HIV-1_{IIIB}	(−)-FTC-resistant[b]	
(−)-FTC	0.30	>90.0	>300
(−)-BCH-189	1.6	>90.0	>56
DDC	0.30	0.30	1.00
DDI	0.70	0.60	0.86
AZT	0.10	0.02	0.20
PFA	148.0	139.0	0.94
TIBO R82150	0.29	0.14	0.48

[a] Drug susceptibilities were determined in MT-2 cells by the MTT-dye reduction method, as described previously (17, 20). Target cells were inoculated with virus at a multiplicity of 0.01 50% tissue culture infective doses per cell. The virus used had a mixture of the Val-184 and Ile-184 mutations. Mean values from triplicate cultures are shown. The variance from the mean was less than 15%. The correlation coefficients for the data were ≥0.95.

[b] Virus obtained following selection in MT-2 cells in the presence of 30 μM (−)-FTC.

ble to AZT, DDI, DDC, PFA, and TIBO R82150 (Table 2). A trend toward increased sensitivity to AZT (mean of fivefold) was noted in three consecutive experiments ($P > 0.05$).

Genetic studies. To investigate the genetic basis for HIV-1 resistance to (−)-FTC and (−)-BCH-189, the full-length coding sequence of RT was PCR amplified from virus-infected cells. Sequence analysis of RT amplified from PBM cells infected with viruses (week 7) resistant to (−)-FTC and (−)-BCH-189 identified a single amino acid change from Met (ATG) to Val (GTG) at position 184 (data not shown). When the virus selected in 30 μM (−)-FTC in MT-2 cells was studied, a Met-184 (ATG)-to-Val (GTA) or -Ile (ATA) change was noted (Fig. 2). The Met-184-to-Ile change was present in four of the five RT clones that were derived from infected cells after passage in 30 μM (−)-FTC. After three additional passages at a higher selective pressure (50 μM), all of the RT clones (five of five) derived from the infected cells had the Met-184-to-Val change. These data suggest an ordered sequence of mutations from ATG (Met-184) to ATA (Ile) to GTA (Val) under the selective conditions used in MT-2 cells.

An amino acid change from Thr (ACA) to Ile (ATA) was also detected at codon 200 in the resistant clones obtained from MT-2 cells as well as in the patient isolate (see below). However, two of five control clones also had the Thr-200-to-Ile change. Variations at codon 200 have also been reported for the HIV-1_{RF} and HIV-1_{MN} strains (24). Thus, the Thr-200-to-Ile change is unlikely to be responsible for the oxathiolane nucleoside resistance. In addition to the coding mutations mentioned above, a number of silent mutations without amino acid changes were observed in PBM cells (including the patient PBM cells; see below). These included changes at codons 196 (GGG to GGA), 204 (GAG to GAA), 205 (CTG to CTA), and 391 (CTA to CTG). These same mutations were also noted in (−)-FTC-resistant virus selected in MT-2 cells, except for the CTA-to-CTG change at codon 391. The significance of these silent mutations is not known.

Studies with a patient isolate. DNA sequencing analysis of HIV-1 RT amplified from the PBM cells obtained from a patient who had undergone sequential treatment with oral AZT for 2 weeks and then (−)-BCH-189 treatment for 4 months demonstrated a mixture of the Met-184-to-Val

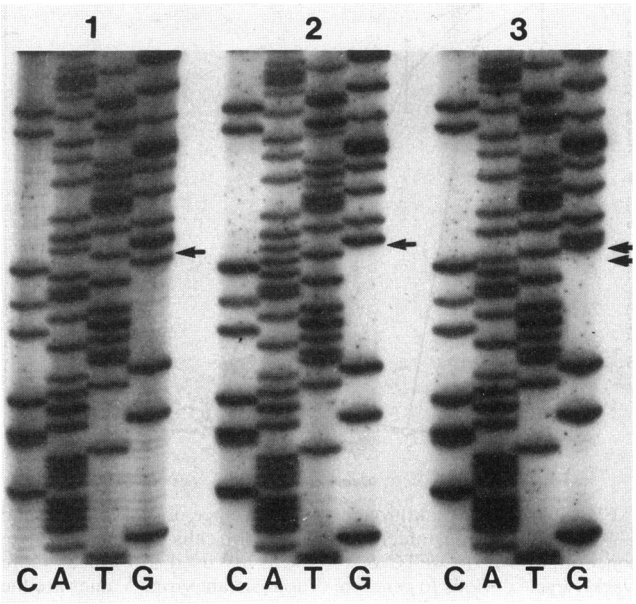

FIG. 2. DNA sequence of cloned RT derived from (−)-FTC-resistant strains showing the Met-to-Val (panel 1, GTA) and Met-to-Ile (panel 2, ATA) mutations at codon 184. The DNA sequence for the parental HIV-1_{IIIB} is shown in panel 3 (ATG). Arrows indicate area of base change. Letters at the bottom of the figure designate the nucleotide within a sequence lane (C, A, T, and G).

(GTG) mutation and the wild-type codon. No mutations commonly ascribed to AZT resistance were noted at codon 41, 67, 70, 215, or 219. Consistent with the mixed mutation at codon 184, virus isolated in the absence of drug selection from the patient's PBM cells was as susceptible as HIV-1_{LAI} to (−)-BCH-189 and AZT when assayed in acutely infected PBM cells (data not shown).

Biochemical studies. Studies were performed to determine the susceptibility of HIV RT derived from drug-resistant virus to FTC-TP, BCH-189-TP, and DDC-TP. The virus particle-derived RTs were obtained from the supernatant of HIV-infected human PBM cells that were known to encode the Met-184-to-Val mutation (week 7). A 15-fold-or-more-decreased susceptibility of these enzymes to FTC-TP or BCH-189-TP was apparent compared with that of parental HIV-1_{LAI} RT (Table 3). However, only a threefold-decreased susceptibility to DDC-TP was noted. Moloney murine leukemia virus RT, which naturally encodes a Val at the position analogous to codon 184 of HIV-1 RT (1), was inherently resistant to FTC-TP and BCH-189-TP and thus behaved like the RT from (−)-FTC- and (−)-BCH-189-resistant viruses. No marked decrease in the susceptibility of the other three enzymes to AZT-TP and PFA compared with that of parental enzyme was noted (Table 3).

DISCUSSION

(−)-FTC and (−)-BCH-189 are new nucleoside analogs with unusually potent activity against HIV-1, HIV-2, simian immunodeficiency virus, feline leukemia virus, and hepatitis B virus in culture with EC_{50}s in the submicromolar range (2, 5, 10, 33, 35). These compounds are more potent and markedly less toxic in certain cells than other related cytidine analogs, such as their (+) enantiomers, DDC, and

TABLE 3. Susceptibility of virion-derived HIV RTs and recombinant Moloney murine leukemia virus RT to oxathiolane-cytosine nucleotides and other antiretroviral agents

RT type	IC_{50} (μM) (fold increase versus HIV_{LAI})[a]				
	FTC-TP	BCH-189-TP	DDC-TP	AZT-TP	PFA
$HIV\text{-}1_{LAI}$ (parental)	0.73	0.34	0.34	0.04	1.13
FTC-resistant HIV-1	17.9 (25)	16.9 (50)	1.10 (3)	0.11 (3)	1.28 (1)
BCH-189-resistant HIV-1	11.2 (15)	9.5 (28)	1.01 (3)	0.16 (4)	1.82 (2)
Moloney murine leukemia virus	15.5 (21)	6.2 (18)	0.99 (2.9)	0.14 (4)	1.34 (1)

[a] The median inhibitory concentration (IC_{50}) was obtained with a $poly(rI)_n \cdot oligo(dC)_{18-24}$ template-primer for the three cytosine analogs as described in Materials and Methods. $Poly(rA)_n \cdot oligo(T)_{12-18}$ was used for the studies with AZT 5′-triphosphate (AZT-TP) and PFA. The experiment was performed in duplicate. The variability of the duplicates was less than 5%. The correlation coefficients for the data were ≥0.98.

3′-fluoro-DDC (5, 9, 35). However, unlike AZT, the oxathiolanes are not active against murine retroviruses.

In the studies reported herein, HIV-1 resistant to (−)-FTC and (−)-BCH-189 emerged within seven weekly passages in human PBM cells at drug concentrations that can be achieved in vivo (31, 40). However, when MT-2 cells were used, (−)-FTC-resistant HIV emerged after two passages in culture. This difference may be related to the higher initial selective concentration of (−)-FTC used in MT-2 cells as well as other factors.

Virus breakthrough was more apparent with (−)-BCH-189 than with (−)-FTC in human PBM cells exposed for 3 weeks to 0.1 μM drug (Fig. 1). This finding is reminiscent of studies in CEM cells which showed that the antiviral activity of (−)-BCH-189 was completely abolished when virus was harvested after a longer incubation time, whereas the potency of (−)-FTC decreased only by a factor of about 10 (35). (−)-FTC- and (−)-BCH-189-resistant viruses were highly cross-resistant to (−)-FTC, (−)-BCH-189, (+)-BCH-189, and (+)-FTC (Table 1), suggesting that all these 3′-thia-compounds interact at the same site on the RT. The results also indicate that the (+) and (−) enantiomers interact in the same way and that there is no stereoselectivity at the binding site in relation to the oxathiolane ring. Interestingly, DDC, a compound structurally related to the oxathiolane nucleosides which contain an endocyclic 3′-methylene group instead of the 3′-thia- group, was not cross-resistant. (−)-FTC- and (−)-BCH-189-resistant viruses were also not cross-resistant to AZT, DDI, FLT, PFA, TIBO R82150, and U-87201E (Tables 1 and 2).

DNA sequencing of the RT gene from (−)-FTC-resistant HIV-1 identified base changes (ATG to GTG, GTA, or ATA) that alter the predicted amino acid residue at position 184 from Met to Val or Ile. It appears that under lower selective pressure in MT-2 cells the Met-to-Ile change can occur with greater frequency than at high selective pressure when the Met-to-Val mutation predominates. In primary human lymphocytes, only the Met-to-Val mutation was identified under the experimental conditions used. With (−)-BCH-189 selection, which was performed only in human PBM cells, only the single base change (ATG to GTG) from Met-184 to Val was noted. The Met-184 residue is conserved in all previously reported HIV-1 isolates (22) and is adjacent to the putative catalytic site of RT composed of the carboxylate triad of Asp-185, Asp-186, and Asp-110 (15). In addition, the Met-Asp-Asp motif is conserved at the putative catalytic site of most other retroviral RTs including those from HIV-2, simian immunodeficiency virus, human T-cell leukemia virus type 1, equine infectious anemia virus, caprine arthritis-encephalitis virus, visna virus, bovine leukemia virus, and Rous sarcoma virus (1). The one reported exception to this is Moloney murine leukemia virus RT, which has a natural Val substitution present at the analogous position. Of interest, we have tested the activity of (−)-FTC against a Moloney murine leukemia virus-based recombinant retrovirus (39) and have found the 90% effective concentration to exceed 100 μM (data not shown). In addition, the Moloney murine leukemia virus RT exhibited an 18- to 21-fold decreased susceptibility to FTC-TP and BCH-189 compared with that of wild-type HIV-1 RT, which was similar to the resistance seen with the RTs from FTC- and BCH-189-resistant viruses (Table 3). These findings, combined with the sequence derived from our resistant strains of HIV-1, strongly implicate the Met-184-to-Val or -Ile substitution in inducing resistance to FTC and (−)-BCH-189. Inhibition of resistant HIV-1 RT by DDC-TP was decreased by only threefold, suggesting that no substantial cross-resistance between DDC and FTC or BCH-189 would be found, which was the case in the antiviral studies (Tables 1 and 2).

The "YMDD" (Tyr-Met-Asp-Asp) motif is considered diagnostic of retroviral RT (1, 4). This sequence is a marker of key regions of secondary structure that fold to form the major part of the palm domain of RT, the major function of which is to hold a divalent metal ion (probably Mg^{2+}) in position for a catalytic role. The Met-184 is in the very heart of the active site where it has ample opportunity to interact with incoming nucleotides. The importance of the Met-184 in oxathiolane analog activity suggests that this class of compounds would be competitive inhibitors and chain terminators as has been previously shown with FTC-TP and BCH-189-TP (35). It is conceivable that these compounds engage in some specific interaction with the Met-184 sulfur atom exploiting the hydrophobic character of sulfur. It is also important to note that both His and Met are known to engage in strong electronic chelating-type interactions with metal atoms. Thus, one cannot rule out a role for these residues which could contribute to the polymerization reaction indirectly by the metal ion. It is interesting that in (−)-FTC- or (−)-BCH-189-resistant RT, the Met-184 is changed to Val or Ile, which maintains the neutral polar character at that position of HIV-1 RT.

When Larder et al. introduced a Met-184-to-Leu substitution into HIV-1 RT by site-directed mutagenesis (19), the virus demonstrated only 5% of the wild-type RT activity. Similarly, Boyer et al. found that a Met-184-to-Tyr mutation causes an 80% reduction of RT activity (4). Of significance is our observation that a Met-to-Val substitution does not appear to reduce the RT activity compared with that of parental RT since virus with this mutation replicated to high titers as determined by RT assays (data not shown). This underscores the power of in vitro drug selection to identify rare variants that have a replicative advantage in the pres-

ence of specific inhibitors. More recently, the Wellcome group introduced the Met-184-to-Val substitution in RT by site-directed mutagenesis, which resulted in a virus with greater than 1,000-fold resistance to (−)-FTC compared with that of parental virus (24). Interestingly, Wainberg's group in Canada recently indicated that the Met-184-to-Val substitution confers DDI and DDC resistance (12), whereas Boucher and colleagues in Holland recently reported that the Met-184-to-Ile change confers (−)-BCH-189 resistance (3). The position 184 mutation apparently confers modest (fivefold) resistance to DDI in vitro and also occurs in some patients undergoing DDI therapy (12). Recently, Wainberg's group was able to select in MT-4 cells HIV-1 variants that are resistant to racemic BCH-189 (11). These viruses were found to be cross-resistant to DDC and DDI. The authors reported that attempts to isolate resistant virus in primary human lymphocytes were not successful. In addition, the genetic basis for the resistance was not provided. At present, we cannot explain the discrepancy between this work and our data with the pure (−) enantiomer of BCH-189, which indicate no significant cross-resistance with DDC or DDI with virus selected in MT-2 and PBM cells. Thus, it appears that susceptibility of viruses to various nucleoside analogs may be dependent on the type of amino acid mutation at the 184 position and also on the technique and cell culture system used to select these viruses.

Of potential significance was the finding of the same Met-184-to-Val substitution in PBM cells from a patient who had been on (−)-BCH-189 treatment for a period of 4 months. This patient's $CD4^+$ cell count markedly increased during the initial treatment phase but declined after 4 months to baseline levels, at which time treatment was discontinued. Sequencing analysis demonstrated the presence of the Met-184-to-Val mutation in addition to the natural codon at position 184. The mixed nature of the isolate probably explains why the virus was still susceptible to (−)-BCH-189. Although these data are from only one patient, they suggest that the mutation conferring the genotype of resistance to (−)-BCH-189 can develop in vivo. The clinical consequence of the Met-184 mutation leading to (−)-BCH-189 resistance is still unclear. Sequential virus isolates from this patient indicate that the genotype reverts to the natural type within a few months. The development of the resistance mutation in vivo with (−)-BCH-189 therapy suggests that combination therapy designed to maximize antiviral effects while minimizing toxicity and diminishing the emergence of drug-resistant strains should be considered for this drug (30). The identification of the new Met-184-to-Val substitution arising in the presence of (−)-BCH-189 and (−)-FTC should permit effective patient monitoring for the development of resistance to these drugs and to design rational drug combinations. In addition, the novel oxathiolane nucleoside resistance mutation further extends the scope of RT mutations which are still compatible with enzyme function. This may help identify key combinations of drug resistance mutations that are lethal for enzyme activity.

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ADDENDUM IN PROOF

Since submission of this manuscript, site-specific mutational analysis and production of infectious recombinant HIV encoding Val^{184} in RT has demonstrated that this mutation is responsible for high-level resistance to (−)-FTC.

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