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Determinants of Individual Variation in Intracellular Accumulation of Anti-HIV Nucleoside Analog Metabolites[▽]

Elijah Paintsil,^{1,2*} Ginger E. Dutschman,² Rong Hu,² Susan P. Grill,² Chuan-Jen Wang,² Wing Lam,² Fang-Yong Li,³ Musie Ghebremichael,⁴ Veronika Northrup,³ and Yung-Chi Cheng²

Departments of Pediatrics¹ and Pharmacology² and Yale Center for Clinical Investigation,³ Yale University School of Medicine, New Haven, Connecticut 06520, and Harvard University School of Public Health, Boston, Massachusetts 02115⁴

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Individual variation in response to antiretroviral therapy is well-known, but it is not clear if demographic characteristics such as gender, age, and ethnicity are responsible for the variation. To optimize anti-HIV therapy and guide antiretroviral drug discovery, determinants that cause variable responses to therapy need to be evaluated. We investigated the determinants of intracellular concentrations of nucleoside analogs using peripheral blood mononuclear cells from 40 healthy donors. We observed individual differences in the concentrations of the intracellular nucleoside analogs; the mean concentrations of the triphosphate metabolite of ethynylstavudine (4'-Ed4T), zidovudine (AZT), and lamivudine (3TC) were 0.71 pmol/10⁶ cells (minimum and maximum, 0.10 and 3.00 pmol/10⁶ cells, respectively), 0.88 pmol/10⁶ cells (minimum and maximum, 0.10 and 15.18 pmol/10⁶ cells, respectively), and 1.70 pmol/10⁶ cells (minimum and maximum, 0.20 and 7.73 pmol/10⁶ cells, respectively). Gender and ethnicity had no effect on the concentration of 4'-Ed4T and 3TC metabolites. There was a trend for moderation of the concentrations of AZT metabolites by gender ($P = 0.17$ for gender · metabolite concentration). We observed variability in the activity and expression of cellular kinases. There was no statistically significant correlation between thymidine kinase 1 (TK-1) activity or expression and thymidine analog metabolite concentrations. The correlation between the activity of deoxycytidine kinase (dCK) and the 3TC monophosphate metabolite concentration showed a trend toward significance ($P = 0.1$). We observed an inverse correlation between the multidrug-resistant protein 2 (MRP2) expression index and the concentrations of AZT monophosphate, AZT triphosphate, and total AZT metabolites. Our findings suggest that the observed variation in clinical response to nucleoside analogs may be due partly to the individual differences in the intracellular concentrations, which in turn may be affected by the cellular kinases involved in the phosphorylation pathway and ATP-binding cassette (ABC) transport proteins.

Individual variation in response, such as viral suppression and adverse effects, to antiretroviral therapy is a well-described phenomenon (19, 49). Epidemiological and limited clinical studies suggest that demographic characteristics (e.g., gender, age, and ethnicity) and the HIV disease state of an individual may be partly responsible for the variation in efficacy and toxicity observed with treatment by nucleoside analog reverse transcriptase inhibitors (NRTIs). For example, published studies show that women experienced a 4-fold lower rate of disease progression than did men while they were on zidovudine (AZT) monotherapy; however, women experienced exaggerated toxicities during NRTI therapy compared to those of men (15–17, 20, 37). Gender and ethnicity have been suggested to be possible variables that explain the observed differences in treatment response to NRTIs (1, 16, 43).

In a cohort of 4 HIV-1-infected women and 29 HIV-1-infected men who initiated AZT, lamivudine (3TC), and indinavir, the triphosphate (TP) levels of AZT were 1.6-fold higher and those of 3TC were 2.3-fold higher in the women than in the men (1). There are limited numbers of studies on intracel-

lular concentrations of NRTIs and treatment response because current methods for the quantification of intracellular NRTI metabolite concentrations are technically and analytically challenging. As the anti-HIV activity of NRTIs depends on the intracellular concentration of the triphosphate metabolite, a reliable assay to determine intracellular concentrations of NRTIs is needed in order to elucidate the mechanism for the association observed between patient characteristics, NRTI concentration, and treatment response.

In the cell, NRTIs are phosphorylated to their triphosphate form (active metabolite) in a stepwise fashion, catalyzed by deoxyribonucleoside kinases, nucleoside monophosphate (MP) kinases (NMPKs), and nucleoside diphosphate (DP) kinases (NDPKs) (47). Phosphoglycerate kinase (PGK) can phosphorylate the diphosphate metabolites of nucleoside analogs such as AZT and ethynylstavudine (4'-Ed4T), a novel inhibitor, to their triphosphate metabolites (24). NRTI triphosphate is incorporated into HIV DNA by HIV reverse transcriptase (RT) and causes termination of HIV DNA chain elongation (27). The potency of NRTIs is dependent on their ability to inhibit the RNA-dependent DNA activity of HIV-1 RT. The adverse effects of NRTIs are mediated by their effects on host DNA polymerase activity. NRTI-induced inhibition of mitochondrial DNA (mtDNA) synthesis is believed to induce depletion of cellular mtDNA and is ultimately responsible for the delayed toxicity (10, 11). Thus, the inhibition of viral RNA

* Corresponding author. Mailing address: Departments of Pediatrics & Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520. Phone: (203) 785-6101. Fax: (203) 785-6961. E-mail: elijah.paintsil@yale.edu.

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replication results in the anti-HIV activities of NRTIs, while the inhibition of host DNA replication results in the clinical toxicities of NRTIs (26, 35).

We hypothesized that patient-dependent variability in intracellular concentrations of NRTI metabolites may be partly responsible for the heterogeneity in virologic suppression and the observed clinical toxicities. In the current study, we used an *in vitro* model of peripheral blood mononuclear cells (PBMCs), target cells for HIV infection, from healthy donors to accomplish the following aims: (i) to investigate whether the variation in response is due to individual differences in the accumulation of intracellular metabolites of nucleoside analogs (4'-Ed4T, AZT, and 3TC); (ii) to identify whether demographic characteristics of patients, such as gender and ethnicity, influence the intracellular concentrations of nucleoside analogs; and (iii) to explore whether the variation in intracellular metabolite concentrations is associated with the activity and/or expression of the cellular kinases involved in the phosphorylation of nucleoside analogs and expression of ATP-binding cassette (ABC) transport proteins.

MATERIALS AND METHODS

Study design. We conducted a series of cross-sectional studies using 40 healthy HIV-seronegative volunteers (20 females and 20 males) from New Haven, CT. The volunteers had no concurrent infections and were not receiving acute or chronic medications. Each participant donated about 40 ml of venous blood. Samples were collected between 9:00 a.m. and 11:00 a.m. to avoid variations due to circadian rhythm. PBMCs were obtained from blood using Ficoll-Hypaque density gradient centrifugation as previously described (46). The PBMCs were stimulated with phytohemagglutinin (PHA; 10 μ g/ml; Sigma-Aldrich Corp., St. Louis, MO) and interleukin-2 (IL-2; 20 U/ml) for 72 h and used in the studies described below. Samples from all 40 participants were used for intracellular concentration studies. However, for the substudies, participants were randomly selected for studies of antiviral activity ($n = 3$), repetition of the intracellular concentration assay ($n = 3$), studies of kinase enzyme activity and expression ($n = 9$), and studies of ABC expression ($n = 9$). The study protocol was approved by the Institutional Review Board of the Yale University School of Medicine. All participants gave their written informed consent before participation in the study.

Chemicals. 4'-Ed4T was synthesized in the laboratory of Hiromichi Tanaka, School of Pharmaceutical Sciences, Showa University, Tokyo, Japan (21). Thymidine (dThd), AZT, stavudine (D4T), and 3TC were purchased from Sigma-Aldrich Corp. The purity of these compounds was verified by high-pressure liquid chromatography (HPLC) analysis. The radiochemicals [*methyl*- 3 H]AZT and [3 H]3TC were purchased from Moravsek Biochemicals Inc. (Brea, CA), and [3 H]4'-Ed4T was a generous gift from Oncolys BioPharma Inc. (Tokyo, Japan). All other chemicals used were of analytical grade or higher.

Assay of anti-HIV activities of nucleoside analogs in CD4⁺ T cells. CD4⁺ T cells were isolated from the PBMCs of 3 out of the 40 healthy donors (1 female and 2 males) using positive selection by anti-CD4-conjugated magnetic beads (Dynabeads M-450 CD4; Dynal, Oslo, Norway) as previously described (34).

The anti-HIV activities of 4'-Ed4T, AZT, D4T, and 3TC were tested in 5×10^5 PHA-stimulated CD4⁺ T cells/ml/well infected with HIV-1 strain IIIB at a multiplicity of infection of 0.1 as described previously (36). In brief, serial dilutions of each analog were placed in triplicate wells of a 24-well tissue culture plate containing 5×10^5 PHA-stimulated CD4⁺ T cells/ml/well in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum and 100 μ g of kanamycin/ml. Five days postinfection, culture supernatant was harvested for HIV-1 RNA extraction using a QIAmp viral RNA extraction kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. HIV-1 RNA extracted from culture supernatant was quantified using real-time RT-PCR with previously published primer set, probes, and cycling parameters (13). The anti-HIV activity was expressed as the concentration of analog producing a 50% inhibition of virus growth (50% effective concentration [EC₅₀]).

Analysis of intracellular concentration of nucleoside analog metabolites in PBMCs. We determined the intracellular concentrations of 4'-Ed4T, AZT, and 3TC metabolites using PBMCs from all 40 healthy donors. PHA-stimulated PBMCs (1×10^7 to 2×10^7 cells/10 ml) were treated with 1 μ M [3 H]4'-Ed4T,

[3 H]AZT, or [3 H]3TC for 24 h, and the intracellular metabolites were quantified using HPLC as previously described (39). In brief, after 24 h of incubation, the cells were harvested by centrifugation and washed twice in ice-cold phosphate-buffered saline (PBS) containing 20 μ M dipyrindimole (Sigma). The cell pellets were extracted with 15% trichloroacetic acid for 15 min on ice. The supernatant containing the NRTI and its phosphorylated metabolites was neutralized by two half-volume extractions with a 45:55 ratio of triethylamine and 1,1,2-trichlorotrifluoroethane. A 50- μ l aliquot of the aqueous phase was injected onto the HPLC column.

Immunostaining for PCNA and TK-1. Aliquots of PBMCs (4×10^4) were spun onto slides using cytospin. Cells were fixed using 4% paraformaldehyde in PBS and were then permeabilized with 0.5% Triton X-100 in PBS. Cells were immunostained using the protocol previously described (35). In brief, the fixed cells were exposed to monoclonal anti-proliferating cell nuclear antigen (anti-PCNA) or anti-thymidine kinase 1 (anti-TK-1) at room temperature for 1 h, followed by fluorescein isothiocyanate-conjugated anti-mouse IgG at a 1:200 dilution. Propidium iodide (PI) was used to stain the nuclear DNA (Molecular Probes, Eugene, OR). Micrographs of the slides were taken using a laser scanning confocal microscope (LSM 510; Carl Zeiss, Inc., Thornwood, NY).

TK-1 and deoxycytidine kinase (dCK) assay. Aliquots of PHA-stimulated PBMCs from nine participants were suspended in extraction buffer (0.01 M HCl [pH 7.5], 3 mM dithiothreitol [DTT], 50 μ M dThd) and stored at -80°C until the enzyme activity assay. The suspension was frozen and thawed three times, and 150 mM KCl was added. The mixture was sonicated three times at 50 W for 5 s each time and centrifuged at 15,000 rpm at 4°C for 30 min. The supernatant from each sample was divided into aliquots, placed into several tubes, and stored at -20°C until enzyme activity assay. The enzyme activity was determined using the DE-81 disc assay (Whatman, Clifton, NJ) as previously reported (12). In brief, the enzyme assay mixture was incubated at 37°C for 30 min. The reaction was stopped by spotting 50 μ l of the reaction mixture on a Whatman DE-81 paper disc, then immediately dropped into 95% ethanol (10 ml/disc), and then washed twice with 95% ethanol. The disc was dried and inserted into a vial containing 7.5 ml of scintillation solution (SafeScint; American Bioanalytical, Natick, MA). Counting of radioactivity was performed in a scintillation counter (Beckman Coulter Inc., Fullerton, CA). The unit of enzyme activity is expressed as 1 nmol product formed per min at 37°C under our standard conditions.

Western blot analysis of phosphorylating enzymes of nucleoside analogs. Expression of the cellular kinases involved in the phosphorylation of nucleoside analogs (i.e., TK-1, dCK, CMP kinase [CMPK], dTMP kinase [TMPK], and PGK) was determined by standard Western blot analysis as previously described using β -actin as a control (39). In brief, whole-cell lysate containing 40 μ g of protein from each donor was electrophoresed through a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham). The membrane was incubated overnight at 4°C with blocking solution (Tris-buffered saline, 0.2% Tween 20, 5% nonfat milk), followed by the specific primary antibody of interest. The membrane was then incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:3,000; Sigma) or anti-rabbit IgG (1:10,000; Sigma), and the signal was visualized using enhanced chemiluminescence (Perkin-Elmer Life Science), according to the manufacturer's instructions. The level of expression was quantified using Pathway software (BD Biosciences, San Jose, CA). The density of the band for enzyme expression was normalized to that of β -actin. The TK-1 concentration was further normalized to that of PCNA since TK-1 expression is cell cycle dependent (7).

Quantitative RT-PCR for expression of ABC transport proteins. RNA was extracted from 400 μ l of PBMCs—comprising 4×10^5 cells, on average—using the TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The concentration of total RNA in each sample was determined with a spectrophotometer. For each sample, 1 μ g total RNA was used for reverse transcription using a cloned avian myeloblastosis virus first-strand cDNA synthesis kit (Invitrogen). Quantitative real-time PCR was performed as previously described (33). In a total volume of 20 μ l reaction mixture, 0.1 μ g of cDNA templates was mixed with 10 μ l of DyNamo HS SYBR green quantitative PCR (qPCR) master mix (MJ Research Inc.) and each pair of primers at a final concentration of 200 nM. The thermal cycling conditions were set up as follows: initial incubation at 50°C for 2 min, denaturation at 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curve analysis was performed after the completion of PCR to assess the possibility of false-positive results. All of the samples were run in duplicate in three independent experiments. The primers for ABC transport genes (MDR1, MRP1, MRP2, and MRP4) have been previously described (14). The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as an internal control for all reactions. The threshold cycle (C_T) values of the genes for each donor were determined. The mRNA expression of ABC transport proteins was calculated as an expression index (EI), derived

from a formula previously described (38): $1,000 \times 2^{-\Delta CT}$, where ΔC_T is the C_T of ABC minus the C_T of GAPDH.

Data analysis and statistics. Continuous data are summarized as means with standard deviations in all tables. Using linear mixed-effects modeling, we examined the effects of the gender and ethnicity of study participants on the intracellular concentrations of nucleoside analog metabolites. The analysis took into account the fact that nucleoside analogs are phosphorylated to the active metabolite (triphosphate) in a stepwise fashion, and, therefore, the product at each step depends on the product of one of the previous steps. Data are presented in the figures as means with standard errors.

The association between intracellular metabolite levels and enzyme activity was examined with the Spearman correlation. Spearman correlation, a nonparametric test to assess statistical association between two variables, was used for the subanalysis due to the relatively small sample size. In all analyses, *P* values are two-sided, with *P* values of less than 0.05 considered statistically significant and *P* values of about 0.10 considered trends. Data were analyzed using SAS software (version 9.2; Cary, NC).

RESULTS

Characteristics of study participants. Forty healthy and HIV-seronegative individuals (20 females and 20 males) were enrolled for the study. Table 1 presents the racial/ethnic characteristics of the study participants. For the analysis, all participants of African descent were categorized as black and the rest were categorized as nonblack (i.e., Caucasians, Asians, and Hispanics). All study participants were adults (i.e., ≥ 18 years of age), except for donors 8 and 34, who were 16 years of age at enrollment (parental consent was obtained for these two donors).

Individual differences in anti-HIV activities of nucleoside analogs. We first determined whether the observed variation in treatment response in HIV-infected individuals could be replicated in our *in vitro* model. CD4⁺ T cells isolated from PBMCs of three donors were infected with HIV-1 IIB, and HIV-1 RNA was quantified. We found that the EC₅₀s of the inhibitors varied among the three donors (Table 2). For example, the EC₅₀s of the four analogs tested were lower in donor 23 than donor 14. The differences in anti-HIV activities between donors 23 and 14 were 5-, >21-, 2-, and 3-fold for 4'-Ed4T, AZT, D4T, and 3TC, respectively. The anti-HIV activity profiles for 4'-Ed4T and AZT in donors 23 and 15 were similar. The anti-HIV activity was also analog dependent. Interestingly, the intracellular concentrations of 4'-Ed4T-TP, AZT-TP, and 3TC-TP obtained at different times for these individuals correlated positively with the observed anti-HIV activity (Table 2).

Individual differences in intracellular concentrations of nucleoside analogs. With the variation in antiviral activity in the three individuals studied, we posit that our *in vitro* assay could likely model the observed clinical variation in response to NRTIs. Next, we investigated whether the variation is due to individual differences in the accumulation of intracellular metabolites. We observed individual differences in the concentrations of intracellular metabolites (mono-, di-, and triphosphates) of the nucleoside analogs, as illustrated in Fig. 1. The mean concentrations of the TP metabolites (the active moiety) of 4'-Ed4T, AZT, and 3TC were 0.71 pmol/10⁶ cells (minimum and maximum, 0.10 and 3.00 pmol/10⁶ cells, respectively), 0.88 pmol/10⁶ cells (minimum and maximum, 0.10 and 15.18 pmol/10⁶ cells, respectively), and 1.70 pmol/10⁶ cells (minimum and maximum, 0.20 and 7.73 pmol/10⁶ cells, respectively). The metabolic profiles of the two thymidine analogs

TABLE 1. Genders and ethnicities of study participants

Donor no.	Gender ^a	Ethnicity ^b
1	M	AD
2	M	AD
3	F	H
4	F	AD
5	M	AD
6	M	AD
7	M	C
8	F	C
9	M	C
10	F	AD
11	M	AD
12	F	C
13	M	C
14	M	AS
15	F	C
16	F	C
17	F	C
18	F	AD
19	M	AD
20	F	AD
21	F	AD
22	M	C
23	M	C
24	M	AD
25	M	AD
26	F	C
27	M	AD
28	F	AD
29	M	C
30	M	AD
31	F	AD
32	F	C
33	M	H
34	F	C
35	F	C
36	F	C
37	F	AD
38	M	C
39	M	AD
40	F	AD

^a M, male; F, female (20 males and 20 females).

^b AD, African descent (*n* = 20); H, Hispanic (*n* = 2); C, Caucasian (*n* = 17); AS, Asian (*n* = 1).

(i.e., 4'-Ed4T and AZT) were qualitatively similar, with the monophosphate metabolite being the predominant metabolite. With 3TC, a cytidine analog, the predominant metabolite was the diphosphate metabolite.

We next investigated whether the observed individual variation is reproducible using PBMCs obtained on a different occasion. We obtained PBMCs to repeat the metabolism experiment for three of the donors at least 3 months after their first assay. As illustrated in Table 3, though with a limited sample size, there was still individual variation in the levels of the intracellular metabolites of 4'-Ed4T. However, there was minimal interday variation in the levels of the metabolites, particularly the level of the triphosphate metabolite. We observed a fold difference of only up to 2.5 in the levels of any of the metabolites between the two time points in an individual.

Traditionally, PBMCs are stimulated with PHA prior to *in vitro* HIV cultivation. We therefore investigated the effect of

TABLE 2. Anti-HIV activities of nucleoside analogs in donor CD4⁺ T cells

Donor no.	Gender ^a	Ethnicity ^b	Mean concn (pmol/10 ⁶ cells) ± SD			EC ₅₀ (μM) ± SD			
			4'-Ed4T-TP	AZT-TP	3TC-TP	4'-Ed4T	AZT	D4T	3TC
14	M	AS	0.13 ± 0.1	0.23 ± 0.1	0.72 ± 0.2	0.166 ± 0.02	0.21 ± 0.01	>0.26	0.151 ± 0.05
15	F	C	0.64 ± 0.2	0.61 ± 0.1	0.61 ± 0.1	0.039 ± 0.004	<0.01 ^c	ND ^d	ND
23	M	C	1.3 ± 0.2	0.36 ± 0.1	2.0 ± 0.2	0.032 ± 0.003	<0.01 ^c	0.121 ± 0.03	0.050 ± 0.004

^a M, male; F, female.^b AS, Asian; C, Caucasian.^c The lowest concentration tested resulted in 24% inhibition in comparison with that in control wells without AZT treatment.^d ND, not determined due to limited cell numbers.^e The lowest concentration tested resulted in 35% inhibition in comparison with that in control wells without AZT treatment.

PHA stimulation on PCNA and TK-1 expression in two individuals. Pre- and post-PHA-stimulated PBMCs from these individuals were immunostained for detection of differences in PCNA and TK-1 expression levels. As anticipated, the expres-

sion levels of PCNA and TK-1 were higher after PHA stimulation, as illustrated in Fig. 2, which presents the results for donor 11 as a representative example. Interestingly, the cell size increased after PHA stimulation. Qualitatively, we did not observe significant differences in the fold changes after PHA stimulation between the two individuals.

Determinants of individual differences in intracellular concentrations of nucleoside analogs. Gender and ethnicity have been suggested to be possible variables that explain the observed differences in treatment response to NRTIs (1, 16, 43). We examined the effects of the genders and ethnicities of the study participants on the intracellular concentrations of nucleoside analog metabolites (Fig. 3). The analysis took into account the fact that nucleoside analogs are phosphorylated to the active metabolite (triphosphate) in a stepwise fashion, and therefore, the product at each step depends on the product of one of the previous steps. We found that there was an overall difference in the amounts of the three metabolites (mono-, di-, and triphosphates), adjusting for the effects of gender and ethnicity. For 4'-Ed4T and 3TC, neither gender nor ethnicity was a significant predictor of the concentrations of their respective metabolites; however, we found a trend for the overall effect of gender on the concentrations of AZT metabolites ($P = 0.07$). We tested for the moderating effects of gender and ethnicity (Fig. 3) on the concentrations of metabolites and found no effect for 4'-Ed4T and 3TC; however, there was a trend for moderation of the concentration of AZT metabolites by gender ($P = 0.17$ for gender · metabolite concentration). When the trajectories of the analog concentrations from monophosphate to triphosphate were analyzed by gender

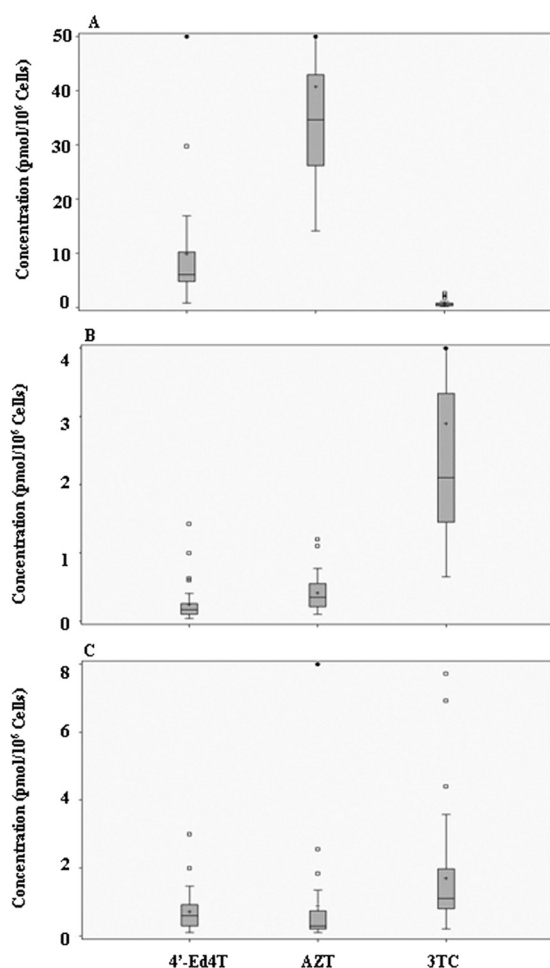


FIG. 1. Individual variation in concentrations of intracellular metabolites of nucleoside analogs. Ranges, medians, and 25% to 75% interquartile ranges of the concentrations of the intracellular metabolites of 4'-Ed4T, AZT, and 3TC in the PBMCs of 40 healthy volunteers are shown. Intracellular concentrations of the analogs were determined using HPLC. Each data point is an average of triplicate assays. ●, data point out of range; +, mean value. (A) Monophosphate metabolites; (B) diphosphate metabolites; (C) triphosphate metabolites.

TABLE 3. Interday variation in the concentrations of 4'-Ed4T intracellular metabolites of three individuals

Donor no. ^a and assay	Ethnicity ^b	Mean concn (pmol/10 ⁶ cells) ± SD		
		4'-Ed4T-MP	4'-Ed4T-DP	4'-Ed4T-TP
9	C			
1st		8.9 ± 0.6	0.3 ± 0.12	0.7 ± 0.03
2nd		6.6 ± 1.8	0.3 ± 0.04	0.6 ± 0.19
11	AD			
1st		3.8 ± 0.02	0.2 ± 0.01	0.4 ± 0.03
2nd		7.1 ± 0.3	0.1 ± 0.01	0.3 ± 0.11
33	H			
1st		5.6 ± 2.59	0.1 ± 0.02	0.5 ± 0.09
2nd		3.2 ± 0.001	0.1 ± 0.02	0.2 ± 0.06

^a All three donors were male.^b C, Caucasian; H, Hispanic; AD, African descent.

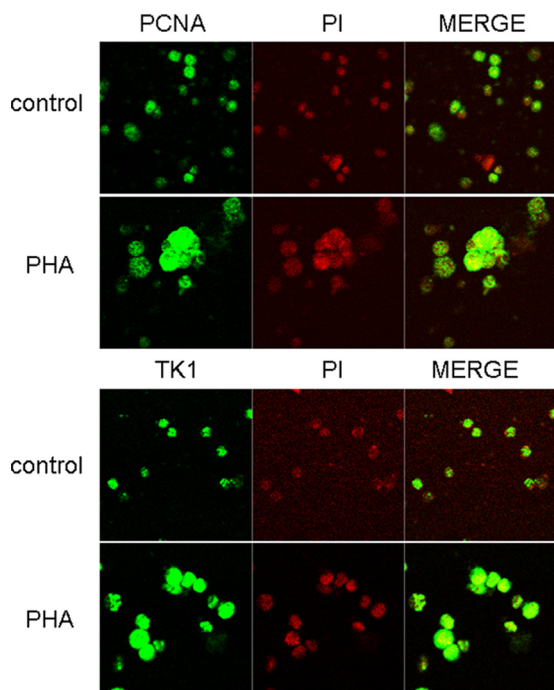


FIG. 2. Effect of PHA stimulation on PCNA and TK-1 expression in donor PBMCs. PBMCs from donor 11 (a male of African descent) were stimulated without PHA or IL-2 (control) and with PHA (10 μ g/ml) and IL-2 for 72 h prior to immunostaining to determine the level of expression of PCNA and TK-1. PI was used to stain the nuclear DNA (second column). Micrographs of the slides were taken using a laser scanning confocal microscope. (Top two panels) PCNA expression without and with PHA stimulation; (bottom two panels) TK-1 expression without and with PHA stimulation.

within each ethnic group, the trend for moderation of the concentration of AZT metabolites by gender remained (data not shown).

Decreased expression of phosphorylating enzymes has been implicated in the observed resistance to nucleoside analogs (5, 25). Thus, we investigated whether the variation in intracellular metabolite concentrations is associated with the activity and/or expression of the cellular kinases involved in the phosphorylation of nucleoside analogs (i.e., 4'-Ed4T, AZT, and 3TC). Nine donors (i.e., five black and four nonblack individuals) were randomly selected for both enzyme activity studies (i.e., TK-1 and dCK) and enzyme expression studies (i.e., TK-1, TMPK, PGK, dCK, CMPK, and PCNA) (Fig. 4). In the nine individuals, we observed individual variations in the association between cellular kinase activity, expression, and formation of metabolites. There was no statistically significant correlation between TK-1 activity and expression of the enzymes involved in the phosphorylation of thymidine analog 4'-Ed4T and AZT metabolites (Fig. 4A, B, and D), except for PGK and 4'-Ed4T-DP (Spearman correlation, 0.86; $P < 0.01$). The correlation between the activity of dCK (dCK is responsible for conversion of 3TC to 3TC-MP) and total 3TC and 3TC-MP metabolite concentrations showed a trend toward significance ($P = 0.1$).

The effective intracellular concentrations of nucleoside analog metabolites depend on the rate of phosphorylation by

cellular kinases and the efflux by transport proteins (e.g., ABC transport proteins). In nine individuals, we investigated whether the expression of ABC transport proteins (MDR1, MRP1, MPP2, and MRP4) was associated with the variation in the intracellular concentrations of the metabolites. After 72 h of PHA stimulation of PBMCs, cells were harvested prior to incubation with radiolabeled nucleoside for RNA extraction and mRNA expression of ABC transport proteins was determined using qPCR. We found no statistically significant correlation between the ABC protein expression index and either gender or ethnicity (Table 4 and data not shown). There was no statistically significant correlation between the expression index of ABC transport proteins and the metabolites of 4'-Ed4T and 3TC. However, we observed an inverse correlation between the MRP2 expression index and AZT-MP, AZT-TP, and total AZT metabolite concentrations, with a borderline significance (Spearman correlations, -0.58 , -0.64 , and -0.58 , respectively; $P = 0.1$, 0.06 , and 0.1 , respectively).

DISCUSSION

In the current study, we observed individual variation in the concentrations of the intracellular metabolites of the nucleoside analogs tested in the study cohort. However, the variation in the metabolite concentrations, including the concentrations of the TP metabolites, a good predictor of anti-HIV activity (1), could not be adequately accounted for by gender or ethnicity. Furthermore, there were differences among the analogs tested. Interestingly, we observed a trend for the effect of gender on the trajectory of AZT metabolite concentrations: $P = 0.07$ for gender and $P = 0.17$ for the interaction of gender and metabolites. That is, AZT metabolite concentrations tended to be higher in the female subjects than in the male subjects. There was no effect of ethnicity on metabolism of nucleoside analogs. Our findings suggest that the observed variation in clinical response to nucleoside analogs, i.e., virologic suppression and toxicity, may be due partly to the individual differences in the intracellular concentrations of NRTIs, which in turn may be affected by the level and regulation of the cellular kinases involved in the phosphorylation pathway and ABC transport proteins.

Nucleoside analogs will continue to be important components of antiretroviral therapy. Yet it should be borne in mind that the HIV reverse transcriptase enzyme can use endogenous deoxynucleoside triphosphates (dNTPs) for HIV DNA synthesis. Thus, nucleoside analog triphosphates compete with endogenous dNTPs in HIV replication, and the dNTP pool size in individuals may vary. Factors that affect treatment variation could be manipulated to optimize HIV therapy, and their identification may be useful in the early phases of drug discovery by influencing the selection of candidate agents for anti-HIV chemotherapy that lessen untoward drug effects. Hence, there is a need to develop sensitive techniques to assess the effect of the endogenous dNTP pool size on the efficacies of nucleoside analogs.

The effects of gender and race on the response to NRTI treatment have been a point of investigation and controversy in diverse studies. Although women were observed to have a better response to AZT monotherapy than men and women experienced exaggerated toxicities during NRTI therapy com-

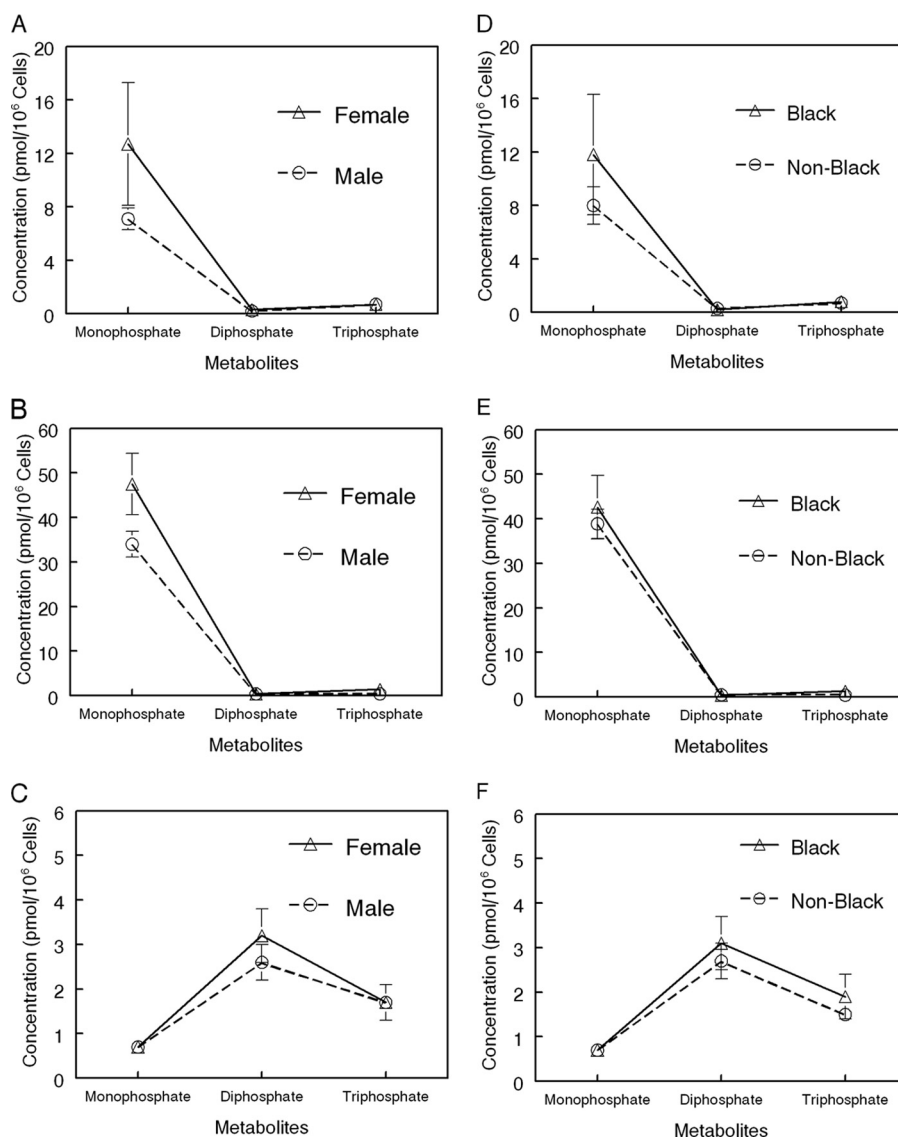


FIG. 3. Relationship between gender or ethnicity and concentrations of intracellular metabolites of nucleoside analogs. The intracellular concentrations of the analogs for the 40 donors were determined using HPLC. Each data point is an average of triplicate assays and is reported as the mean value $\pm$ standard error of the mean. (A to C) Results for 4'-Ed4T (A), AZT (B), and 3TC (C) by gender; (D to F) results for 4'-Ed4T (D), AZT (E), and 3TC (F) by ethnicity.

pared to those experienced by men (15–17, 20, 37), earlier trials in the era of AZT monotherapy failed to demonstrate a gender difference in rates of disease progression (31). However, in these early trials women comprised less than 10% of the study participants (18, 41, 48). The first study (ACTG 175) preplanned with the power to detect a gender difference ultimately found differences in the pretreatment characteristics of study participants, confounding the observed treatment effect (16). The study investigators postulated a plausible gender difference in response to antiretroviral therapy; this has since been a subject of several investigations. Our findings support the possibility of an effect of gender on nucleoside analog efficacy with regard to AZT, as was observed in earlier clinical studies.

Because NRTIs are activated and exert their anti-HIV activity in the cells, appropriate studies to answer the question of

the effect of gender on the antiviral activity of a nucleoside analog should measure the intracellular concentrations of metabolites. Due to the technical and analytical difficulties in measuring the concentrations of intracellular metabolites, there have been a limited number of studies in this area. Consistent with our findings, some studies have shown an association between gender and levels of intracellular metabolites of NRTIs (1). Some of these studies report higher rates of toxicity and effective antiviral response in women than in men (1, 22, 44), while others report the opposite (3, 6). In other studies, response rate and survival were not dependent on gender but, rather, were dependent on individualized dose adjustments (9, 43). These conflicting reports may be due to limitations of the studies, such as small sample sizes and methodological differences.

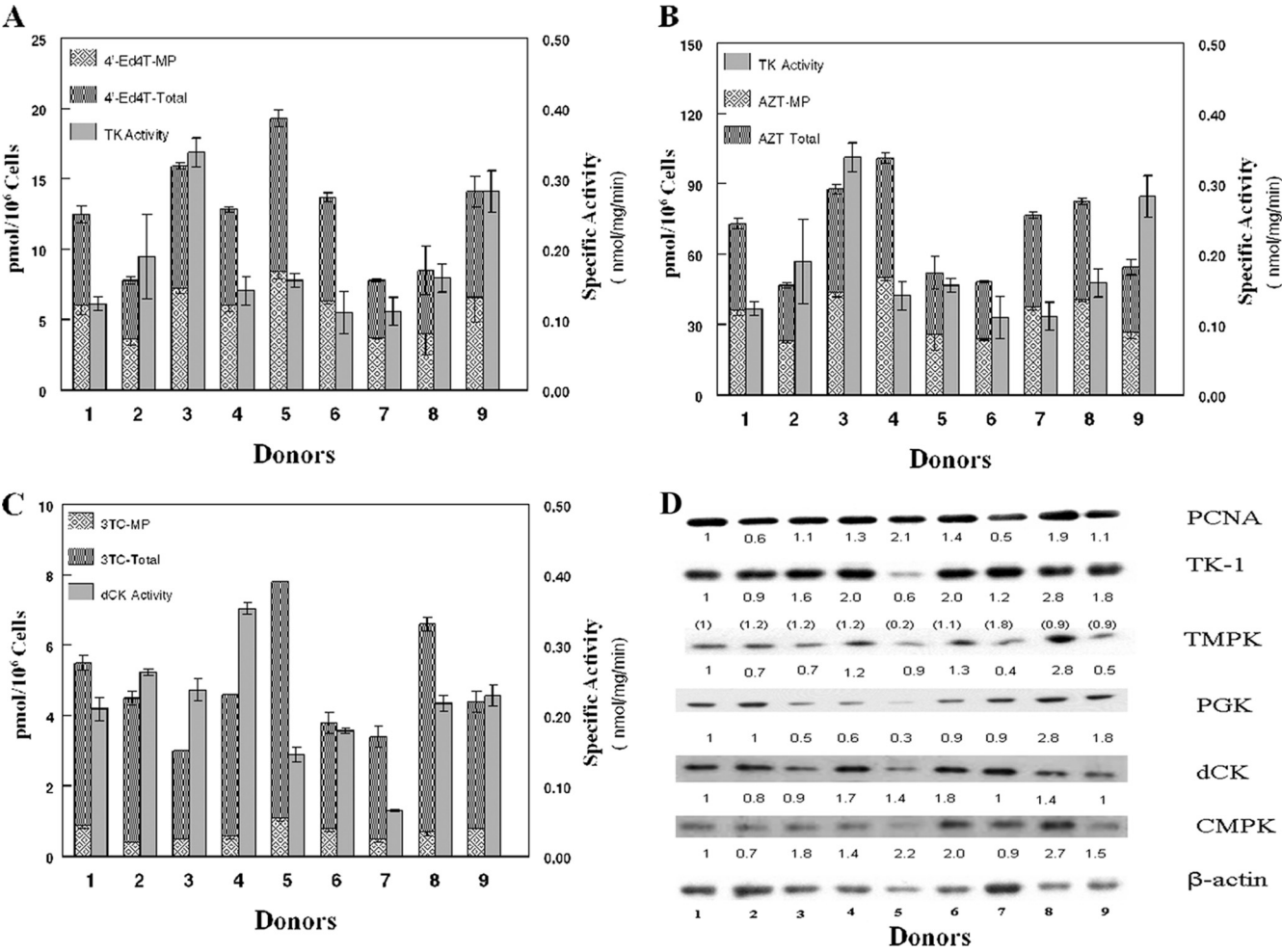


FIG. 4. TK-1 and dCK activities, cellular kinase expression, and intracellular nucleoside analog metabolite levels. (A) TK-1 activities for nine donors with respect to 4'-Ed4T-MP and total 4'-Ed4T metabolites formed. TK-1 activity is the mean of two independent experiments done in duplicate. The results for metabolites are means of an experiment done in triplicate. Data represent means $\pm$ standard deviations. (B) TK-1 activities for nine donors with respect to AZT-MP and total AZT metabolites formed. Metabolite concentrations are means of an experiment done in triplicate. Data represent means $\pm$ standard deviations. (C) dCK activities for nine donors with respect to 3TC-MP and total 3TC metabolites formed. dCK activities are the means of two independent experiments done in duplicate. Metabolite concentrations are means of an experiment done in triplicate. Data represent means $\pm$ standard deviations. (D) Western blot analysis of cellular kinases (TK-1, TMPK, PGK, dCK, CMPK) in cell lysates from nine donors, with β -actin as the control. The density of the band for enzyme expression was normalized to that of β -actin. The number below each band represents the ratio of cellular kinase expression to β -actin expression compared to that for donor 1. The TK-1 concentration was further normalized to that of PCNA since TK-1 expression is cell cycle dependent. The numbers in parentheses under the TK-1 bands are the ratios of TK-1 expression to PCNA expression.

Taken together, as with all biological systems, it is likely that the pathogenesis of the observed intracellular concentrations of NRTIs and treatment effects may be rather complex, and it remains unclear if gender plays a part in this causal pathway *in*

vivo. It is reasonable to posit that variation in nucleoside analog metabolism may be affected by one's race/ethnicity, since the frequencies of certain drug metabolism isoenzymes and allelic variants of efflux transport proteins vary by race/ethnicity (4, 28, 29). Moreover, race has been identified to be an independent risk factor for the NRTI-associated toxicity (32). However, no ethnic difference in the concentrations of intracellular metabolites of NRTIs has been reported to date. Our finding that there is no association between ethnicity and intracellular accumulation of NRTIs is consistent with that of others (44).

The effective concentration of intracellular metabolites of NRTIs will depend on a balance between the efficiency of phosphorylation and efflux of the metabolites. The phosphorylation of NRTIs occurs within cells, and therefore, cellular factors such as cellular kinases and efflux transporters may be

ABC transport protein	EI of mRNA				<i>P</i> value
	Female (<i>n</i> = 5)		Male (<i>n</i> = 4)		
	Mean ± SD	Range	Mean ± SD	Range	
MDR1	2.49 ± 1.52	0.88–4.98	3.84 ± 1.86	2.25–6.48	0.27
MRP1	41.74 ± 12.36	29.46–59.66	58.54 ± 19.77	31.79–76.70	0.16
MRP2	0.40 ± 0.20	0.12–0.65	0.48 ± 0.11	0.38–0.62	0.47
MRP4	1.29 ± 0.52	0.88–2.19	1.66 ± 0.71	1.12–2.61	0.40

critical determinants of the intracellular pharmacokinetics (23). In an analysis of nine study participants, we observed individual variability in the activities of cellular kinases, consistent with the findings of earlier studies (8, 40, 45). We also observed variability in the phosphorylation of different thymidine analogs (AZT and 4'-Ed4T) in some individuals. This may be due to the fact that the substrate specificity of the kinases at each phosphorylation step may be different or that more than one enzyme may be responsible for phosphorylation at each step (24, 39). Turriziani et al. also observed individual variability in TK-1 activity, though the variability was more marked in HIV-infected individuals than in healthy individuals (45). In contrast, Jacobsson et al. observed lower TK-1 activity in stimulated PBMCs of HIV-infected patients than in PBMCs of HIV-noninfected individuals (25). In our study, there was no significant relationship between TK-1 activity and the levels of monophosphate or total metabolites formed. Thus, TK-1 activity may be critical but not necessarily sufficient to predict the final concentrations of metabolites. In the analysis of the correlation of cellular kinase activity, expression, and formation of metabolites in nine subjects, there was no apparent association between enzyme activity, expression, and product formation.

Efflux of metabolites can also affect the final concentrations of metabolites in the cells. The expression and activity of the ABC transport protein MRP4 have been associated with AZT-MP and 3TC-MP concentrations and clinical effect (30). Moreover, the overexpression of MRP4 has been associated with reduced accumulation of AZT-MP within cells (42). In this study, using an mRNA expression index for the ABC transport proteins, there was no statistically significant correlation between the level of MDR1, MRP1, MRP2, or MRP4 and 4'-Ed4T and 3TC metabolite concentrations. However, there was an inverse relationship between the MRP2 expression index and AZT-MP, AZT-TP, and total AZT metabolite concentrations, with borderline significance. We did not observe any association between MRP4 and AZT metabolite concentrations. In the subanalysis of the activities of cellular kinases and expression of ABC transport proteins, there were only nine subjects. Therefore, one has to be cautious in the interpretation of the data. However, our findings and previous reports suggest that at the cellular level there may be a rather complex interplay of multiple factors that are responsible for the intracellular accumulation of nucleoside analog metabolites (2).

Our study, like all *in vitro* studies, has some inherent limitations. We did not take into account the menstrual cycle (i.e., changes in sex hormones) of the female participants. Because we could not process samples from all 40 participants at one time, each sample was processed at the same time of the day (between 9:00 a.m. and 11:00 a.m.) to minimize diurnal differences. Though the interday variation of 4'-Ed4T metabolite concentrations of three of the participants who donated blood on two different occasions at least 3 months apart was minimal (Table 3), our study was not powered to adequately address this issue. Also, the effect of PHA stimulation on the variation of intracellular metabolite concentrations should be minimal, as all donor PBMCs were stimulated with the same concentration of PHA. Another limitation of the study with regard to the effects of cellular kinases or ABC transport proteins, if any, on

intracellular accumulation of metabolites is the small sample size used in the subanalysis.

In conclusion, we observed individual and analog-dependent variations in intracellular concentrations of nucleoside analogs and a possible effect of gender on the metabolism of AZT in particular. The possibility that cellular kinases and ABC transport proteins account for individual variations in the concentrations of nucleoside analogs is provocative and compelling enough to warrant further research to better define those cellular factors that may be responsible for treatment variations.

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Y.-C.C. is a coinventor of the anti-HIV activity of 4'-Ed4T. None of the rest of us declares a conflict of interest.

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