

# Monitoring of didanosine and stavudine intracellular trisphosphorylated anabolite concentrations in HIV-infected patients

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**Objective:** To determine the concentrations of intracellular active anabolites of stavudine (d4T) and didanosine (DDI) and their interpatient variability in HIV-infected patients and to explore relationships between plasma and intracellular forms.

**Methods:** This pilot study included 28 antiretroviral-naïve HIV-infected patients who received d4T (40/30 mg twice daily), ddl (400/250 mg daily) and efavirenz (600 mg daily). After 6 months of therapy, 7 ml of blood was collected between 0.5 and 16.2 h and 2.5 and 28.5 h after the last dose of d4T and ddl, respectively. Plasma samples were obtained for the determination of d4T and ddl concentrations. Peripheral blood mononuclear cells were prepared for measuring intracellular d4T and ddl triphosphates (d4T-TP and ddA-TP, respectively).

**Results:** d4T-TP and ddA-TP concentrations were above the limit of quantification in 25 of 26 compliant patients: median d4T-TP was 31 fmol/10<sup>6</sup> cells (range, 0–99) and median ddA-TP was 8 fmol/10<sup>6</sup> cells (range, 0–23). The half-life of d4T-TP was calculated as 7 h. Interpatient variability in d4T-TP and ddA-TP concentrations was 48% and 58%, respectively. A significant relationship was observed between plasma d4T and intracellular d4T-TP. No relation was found between ddl and ddA-TP. A linear relation was observed between the intracellular concentrations of d4T-TP and ddA-TP.

**Conclusion:** This is the first time that data have been obtained on intracellular concentrations of d4T-TP and ddA-TP, their intracellular pharmacokinetics and interpatient variability. Other similar studies with more patients are needed to enhance knowledge of the intracellular pharmacology of the nucleoside reverse transcriptase inhibitors.

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## Introduction

Nucleoside reverse transcriptase inhibitors (NRTI) are prodrugs that need to be intracellularly phosphorylated to their active form (the trisphosphorylated nucleotide)

to exert their antiviral activity. The trisphosphate form competes with the corresponding endogenous triphosphate nucleotide for binding to viral reverse transcriptase and stops elongation of viral DNA [1,2]. Intracellular metabolic parameters influence phosphor-

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ylation of NRTI (rate-limiting phosphorylation steps [2–4], cell activation status [5,6], infection status [7], prolonged NRTI exposure [8–10]) and lead to a marked interpatient variability in the concentration. Knowledge of the intracellular concentrations of these anabolites is required to improve drug dosing and for understanding their efficacy and toxicity.

The main *in vivo* studies of intracellular pharmacology of NRTI were carried out with patients treated with zidovudine or lamivudine [11–13] and indicated that the clinical efficacy could be related to the intracellular concentration of the active form and not the plasma concentration of the parent drug. Therapeutic drug monitoring would not be possible based on the plasma concentration of nucleoside because no correlation was found between plasma levels and the intracellular pharmacokinetics of the triphosphate anabolite for either zidovudine or lamivudine [14–16].

A direct liquid chromatography tandem mass spectrometry (LC/MS/MS) assay has recently been developed by our group [17–19] to measure intracellular triphosphates of several NRTI, such as stavudine (d4T), didanosine (ddI), lamivudine and zidovudine, in human peripheral blood mononuclear cells (PBMC). A full validation of the simultaneous assay of the triphosphate derivatives of d4T (d4T-TP) and ddI (ddA-TP) has been performed [20] and this assay can produce data with the highest precision and accuracy. The aims of the present study were first to verify the relevance of this assay for d4T-TP and ddA-TP in several clinical samples from d4T- and ddI-treated patients and, for the first time, to investigate interpatient variability and the half-life of elimination. The relationships were also examined between plasma concentrations of the parent drug (d4T and ddI) and the intracellular concentrations of d4T-TP and ddA-TP and also that between d4T-TP and ddA-TP.

## Materials and methods

### Materials and reagents

The phosphorylated anabolites d4T-TP and ddA-TP were provided by Bristol-Myers-Squibb. 2-Chloroadenosine 5'-triphosphate (CIA-TP) was from Sigma (St Louis, Missouri, USA).

CR312 (Jouan, St Herblain, France) and Sigma 2K15 (Bioblock Scientific, Illkirch, France) centrifuges were used for Ficoll cell separation with cell preparation tubes (Vacutainer CPT; Becton Dickinson, Le Pont de Claix, France). *N,N'*-Dimethylhexylamine was from Sigma Aldrich (Milwaukee, Wisconsin, USA). Eppendorf 1.5 ml safe lock cones were used in sample handling (Eppendorf, Germany). Human blood from

healthy subjects was obtained from EFS (Rungis, France) or Biomédia (Boussens, France). Nitrogen HP45 was from Air Liquide (Paris, France). Ultrapure water was from a Milli-Q<sub>plus</sub> 185 purifier (Millipore, Mollshiem, France), gradient grade methanol from Merck (Darmstadt, Germany), high-pressure liquid chromatography (HPLC) quality acetonitrile from SDS (Peypin, France), and formic acid ammonium salt and analytical formic acid from Sigma.

### Instrumentation

An HPLC system 1100 (Agilent Technology, Les Ulis, France) connected to an API 3000 tandem mass spectrometer with an electrospray source (Sciex, Applied Biosystem, France) was monitored using Analyst v. 1.1 acquisition and data treatment software (Applied Biosystem).

### Standard solutions

Stock solutions of d4T-TP and ddA-TP were prepared by precisely weighting an amount corresponding to 1 mg powder, which was dissolved in 10 ml of ultrapure water. Stock solutions and diluted solutions were stored at  $-20^{\circ}\text{C}$ . Standards used for the calibration curve and quality control were also prepared in ultrapure water and stored at  $+4^{\circ}\text{C}$  for 1 week without degradation. Purity of the solution was checked before use using the same chromatographic system coupled with ultraviolet detection (260 nm).

### Preparation of standards and quality controls

PBMC were prepared as previously described [18]. Briefly, approximately 8 ml of drug-free blood (EFS) was transferred to cell preparation tubes and centrifuged at room temperature ( $18-25^{\circ}\text{C}$ ) for 25 min at  $1500-1800 \times g$ . PBMC were collected and washed twice using 0.9% NaCl solution at  $4^{\circ}\text{C}$ . Samples containing  $7-10 \times 10^6$  cells/tube were sampled and stored at  $-20^{\circ}\text{C}$  for utilization for standards and quality controls.

Just before analysis, internal standard (Cl-ATP from Sigma-Aldrich, 20  $\mu\text{l}$ , 60 ng/ml) and d4T-TP and ddA-TP standards were added as calibrators (final d4T-TP: 61.45, 123.0, 1024, 1639, 2253 and 3073 fmol/sample; final ddA-TP: 53.39, 106.8, 889.9, 1424, 1958 and 2670 fmol/sample) and as quality controls (final d4T-TP: 164, 1331 and 2458 fmol/sample; final ddA-TP: 142, 1157 and 2136 fmol/sample). PBMC extract was prepared as previously described [17]. Briefly, PBMC were lysed in 500  $\mu\text{l}$  Tris HCl (0.05 mol/l pH 7.4) methanol (30/70 v/v) buffer maintained at  $-20^{\circ}\text{C}$ . After methanol evaporation, a 40  $\mu\text{l}$  fraction of the remaining solution was injected into the LC/MS/MS system.

### Samples from patients

Adult HIV-1-infected naive patients with advanced immunodeficiency were enrolled in an efficacy and

safety open pilot study in Dakar, Senegal (IMEA 012-ANRS1206) and were treated with d4T (40/30 mg twice daily, in the morning and at bedtime), ddI (400/250 mg daily at bedtime) and efavirenz (600 mg daily at bedtime). After 6 months, 7 ml blood was collected (based on the primary end-point of the study) at a single sampling time within the framework of normal laboratory monitoring in the morning: 0.5–16.2 h and 2.5–28.5 h after the last dosing for d4T and ddI, respectively. PBMC were prepared and treated as described above [18]. Blood was also collected into ethylenediaminetetraacetic acid-treated tubes and centrifuged to collect plasma for analysis of unchanged drug d4T and ddI.

Samples were available for 28 patients (12 male). At baseline, 46% were classed as Centers for Disease Control and Prevention stage C and mean weight was 54 kg (SD, 8), mean viral load was 5.5 log<sub>10</sub> copies/ml (SD, 0.4) and mean CD4 cell count was 134 × 10<sup>6</sup> cells/l (SD, 86). At 6 months, mean viral load was 2.3 log<sub>10</sub> copies/ml (SD, 1.0) and mean CD4 cell count was 226 × 10<sup>6</sup> cells/l (SD, 153). Viral load remained > 200 copies/ml in six patients.

### Chromatographic and mass spectrometric conditions for intracellular analyses

Intracellular analyses were performed at the Pharmacology and Immunology Unit of CEA (France) as previously described [18]. Briefly, chromatography was performed on Supelcogel ODP-50 5 µm, 150 × 2.1 mm (Supelco, St Quentin-Fallavier, France). Mobile phase comprising N',N'-dimethylhexylamine, ammonium formate and acetonitrile gradient grade was delivered at a flow rate of 0.3 ml/min. Mass spectrometer conditions were as previously described [18]. The method was fully validated [20]. Briefly, the lower limit of quantification was established as 61 fmol/sample for d4T-TP and 53 fmol/sample for ddA-TP. Interpatient variability (coefficient of variation) for precision was 7.5–13.4% for d4T-TP and 8.2–11.3% for ddA-TP. Accuracy ranged between –1.5 and 9.6% for d4T-TP and between –15.1 and –5.2% for ddA-TP.

After LC/MS/MS analysis, the result obtained from the calibration curve is given in femtomoles. This result was related to the number of cells as previously determined by biochemical DNA detection. Briefly, after pellet digestion overnight with 1 mol/l sodium hydroxide, the extract was diluted with phosphate buffer and SYB green (S-7525, Molecular Probes, Eugene, Oregon, USA) was used as intercalant. Fluorescent excitation was at 465 nm and emission at 530 nm. This cell counting is based on the linear relationship constructed between cell counting and the fluorescence signal from the DNA content in the pellet.

### Chromatographic and mass spectrometric conditions for plasma analyses

The methods were developed and validated at Bristol-Myers Squibb, Saint-Nazaire, France according to the procedures followed by Clinical Discovery and Analytical Sciences (CDAS; New Brunswick, New Jersey, USA). Two separate methods were validated, one for ddI and one for d4T. The methods involved a solid-phase extraction using an LMS cartridge from Varian (Palo Alto, California, USA) (d4T) or an OASIS cartridge from Waters (France) (ddI) using a 0.5 ml (d4T) or 0.1 ml (ddI) plasma sample. Labeled d4T (D4-d4T) or BMS-180194 (lobucavir, a nucleoside analogue) were used as internal standards for d4T and ddI, respectively. Following liquid chromatography operating in a gradient mode (0.1% formic acid/water/acetonitrile) on an Uptisphere ODB 50 mm × 2.0 mm, 3 µm (Interchim, Montluçon, France), detection was by positive electrospray mass tandem spectrometry. The standard curves ranged from 0.5 ng/ml (LLQ) to 1000 ng/ml and were fitted to 1/x<sup>2</sup> linear regression model. The clinical samples (stored at approximately –20°C) were typically analysed with a fresh daily calibration curve and nine quality control samples.

### Determination of intracellular half-life of stavudine triphosphate

Intracellular d4T-TP concentrations were plotted against time using the pharmacokinetics Software Kinetics 3.0 (InnaPhase, Philadelphia, Pennsylvania, USA) for half-life of elimination between the first and the last samples.

## Results

Intracellular concentrations for the 28 patients are presented in Figs 1 and 2. The d4T-TP concentrations were above the limit of quantification (6.1 fmol/10<sup>6</sup> cells) in 25 out of 28 patients; the ddA-TP

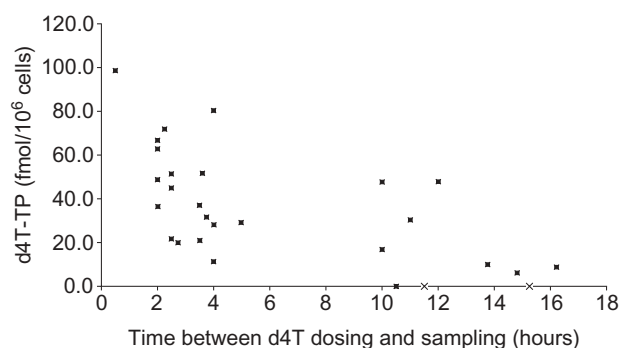
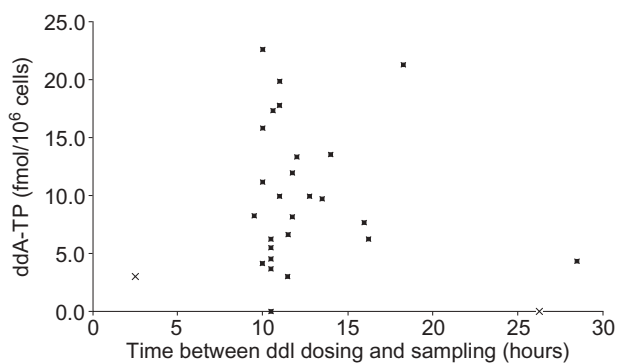


Fig. 1. Changes in stavudine triphosphate (d4T-TP) concentrations with time between dosing and sampling. ■, compliant patients; ×, non-compliant patients.



**Fig. 2. Changes in the intracellular triphosphate anabolite of didanosine (ddA-TP) with the time between dosing and sampling.** ■, compliant patients; ×, non-compliant patients.

concentrations were above the limit of quantification (5.3 fmol/10<sup>6</sup> cells) in 26 out of 28 patients, respectively. The median concentrations of d4T-TP and ddA-TP were 31 fmol/10<sup>6</sup> cells (range, 0–99) and 8 fmol/10<sup>6</sup> cells (range, 0–23) (Figs 1 and 2). Two patients with undetectable d4T-TP and ddA-TP (or with ddA-TP concentration very close to the limit of quantification) had discontinued their treatment following medical assessment, confirmed by high viral load and undetectable plasma d4T and ddI. Undetectable d4T-TP and ddA-TP levels in these two patients were not included in the following analysis.

Since samples were collected in the morning and d4T dosing was also in the morning, whereas ddI dosing was in the previous evening, most of the samples were collected 2–4 h after the last d4T dose and 9–17 h after the last ddI dose. In the 16 samples collected 2–4 h after last d4T dose, which could be expected to be close to the time of maximum plasma concentration, median d4T-TP intracellular concentration was 41 fmol/10<sup>6</sup> cells (range, 11–81) and median d4T plasma concentration was 282 ng/ml (range, 47–669). In the 25 samples collected 9–19 h after the last ddI dosing, median ddA-TP intracellular concentration was 9.7 fmol/10<sup>6</sup> cells (range, 0–23) and median ddI plasma concentration was 23.4 ng/ml (range, 2–161). Interpatient variability in intracellular concentrations was 48% for d4T-TP 2–4 h after dosing and 58% for ddA-TP 9–19 h after dosing. Interpatient variability was 57% and 98% for plasma d4T and ddI concentrations, respectively.

The half-life of elimination of d4T-TP was estimated to be 7 h for d4T-TP but could not be calculated for ddA-TP since most samples were collected between 9 and 17 h after dosing and no lowering of concentrations was apparent.

In the 22 out of 28 patients with a viral load < 200 copies/ml at 6 months, the median d4T-TP intracellular

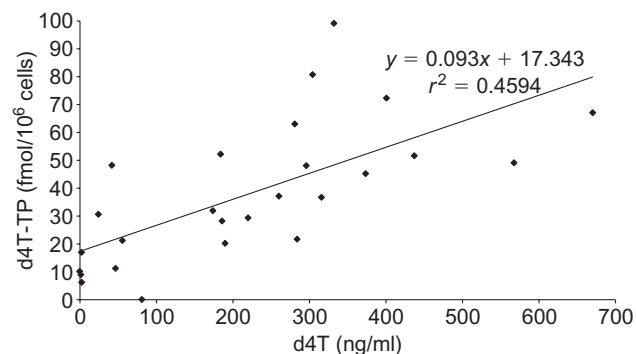
concentration was 34 fmol/10<sup>6</sup> cells (range, 0–99) and that of ddA-TP was 8.2 fmol/10<sup>6</sup> cells (range, 0–23). Two of the six patients with a viral load > 200 copies/ml had discontinued their treatment and the other four had median d4T-TP and ddA-TP concentrations of 36 and 13 fmol/10<sup>6</sup> cells, respectively. Exclusion of these four patients did not significantly change the median and interpatient variability of d4T-TP and ddA-TP intracellular concentrations.

Median intracellular ddA-TP concentrations were similar for the 14 patients (< 60 kg) receiving 250 mg ddI (9.7 fmol/10<sup>6</sup> cells) and for the 12 (> 60 kg) receiving 400 mg (9.9 fmol/10<sup>6</sup> cells). Median d4T-TP intracellular concentrations reached 37 fmol/10<sup>6</sup> cells in the 13 patients (< 60 kg) receiving 30 mg d4T and 50 fmol/10<sup>6</sup> cells in the 12 patients (> 60 kg) receiving 40 mg d4T.

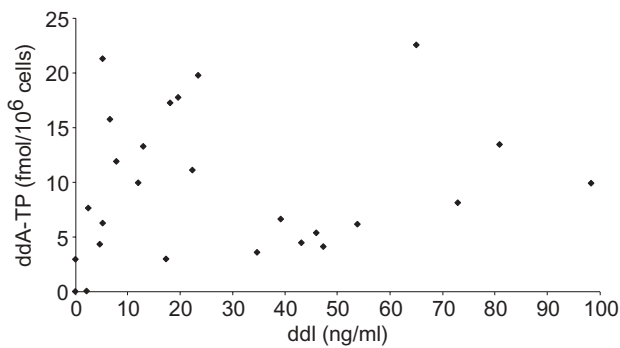
A significant correlation (Fig. 3) was found between intracellular d4T-TP concentrations and plasma d4T concentrations ( $r^2 = 0.46$ ;  $P = 0.0001$ ;  $n = 26$ ) taking into account the whole range of samples (the two non-compliant patients were excluded). A similar correlation ( $r^2 = 0.41$ ;  $P = 0.008$ ;  $n = 16$ ) was found taking only the 16 patients whose samples were collected 2–4 h after the last d4T dose (data not shown). In contrast, no correlation was found for ddA-TP and ddI (Fig. 4).

No correlation was found between intracellular ddA-TP or d4T-TP concentrations with either viral load or CD4 cell count difference between baseline and 6 months or 3 months and 6 months (data not shown).

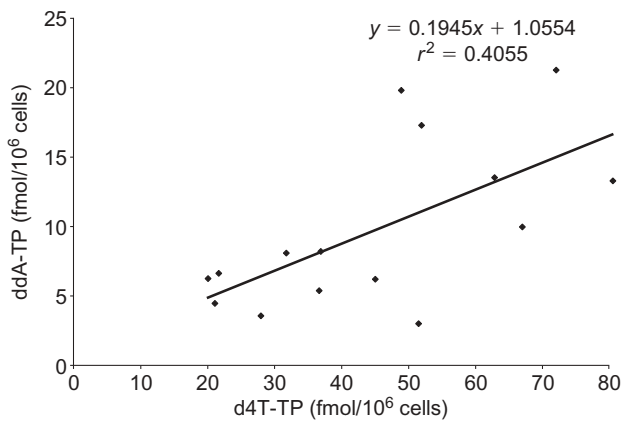
A correlation between intracellular ddA-TP and d4T-TP concentrations was observed ( $r^2 = 0.41$ ;  $P = 0.01$ ) with the 15 samples collected 2–4 h after the last d4T dose and 9–19 h after the last ddI dose (Fig. 5). In contrast, no correlation was found when all samples



**Fig. 3. Linear correlation between plasma stavudine (d4T) and intracellular stavudine triphosphate (d4T-TP) concentrations.**



**Fig. 4. Lack of linear correlation between plasma didanosine (ddl) and its intracellular trisphosphate anabolite (ddA-TP) concentration.**



**Fig. 5. Linear correlation between intracellular stavudine trisphosphate (d4T-TP) and the intracellular trisphosphate anabolite of didanosine (ddA-TP).** Samples collected 2–4 h after last stavudine dose and 9–19 h after last didanosine dose.

were used whatever the sampling time (data not shown).

## Discussion

This study is the first where intracellular concentrations of trisphosphorylated anabolites of ddI (i.e., ddA-TP) and d4T (i.e., d4T-TP) were determined during a clinical trial. Only one patient was found to be under the limit of quantification for ddA-TP and d4T-TP if patients who were considered non-compliant were excluded. Intracellular concentrations remained detectable even when time between dosing and blood sampling was greater than 12 h for d4T-TP and 24 h for ddA-TP. This showed the relevance of the analytical method for measuring active forms of ddI and d4T during clinical trials and also for pharmacokinetic studies. It is worth noting that the intracellular d4T-TP and ddA-TP concentrations determined during this study (median for d4T-TP and ddA-TP was 31 and

8 fmol/10<sup>6</sup> cells, respectively) were in the range of those previously reported using different analytical techniques: quantification of the nucleoside by LC/MS/MS after dephosphorylation of d4T-TP (6.3–22.8 fmol/10<sup>6</sup> cells in two patients [21]) or direct LC/MS/MS (8.3–40 fmol/10<sup>6</sup> cells in four patients [18]). Previous ddA-TP concentrations determined with direct LC/MS/MS in three patients were 6.8–39 fmol/10<sup>6</sup> cells [18].

PBMC were chosen as the target cells as they have been extensively used for such studies since they represent a majority of lymphocytes and can also be easily prepared. However, phosphorylation could differ according to the type of cell (i.e., lymphocytes and monocytes) or the percentage of activated cells, which could be related to infection status. These points have not been considered in this pilot study. Although no differences in phosphorylation have been reported between ethnic groups, it should be kept in mind that this study was performed in African patients.

An elimination half-life of 3.5 h for d4T-TP has been determined in vitro in human PBMC [22]. Our preliminary in vivo half-life of 7 h must be confirmed with more appropriately designed studies, using several samples collected from the same patient at different times. An intracellular half-life of elimination of 25–40 h for ddA-TP has been determined in vitro in MOLT-4 cells [23]. Since no decrease in concentration was noted between 9 and 19 h, this suggests a very long half-life of elimination in vivo for ddA-TP.

Previous determinations of d4T-TP were for a very small number of patients with variable combination therapy and history of treatment, and this could increase interindividual variability of intracellular concentrations, as shown for d4T-TP [8]. In the present study, all patients were drug naive at the start of the study, received the same therapy (ddl, d4T and efavirenz) and were sampled 6 months after baseline. This should allow an accurate determination of interpatient variability. This was 48 and 58% for d4T-TP and ddA-TP, respectively, for concentrations determined after reducing the interval between dosing and blood sampling (2–4 h for d4T-TP and 9–19 h for ddA-TP). The interindividual variability observed for intracellular concentrations of d4T-TP and ddA-TP appeared to be lower than that for plasma d4T and ddl concentrations, probably because of the shorter half-life for plasma concentrations (2 h for d4T, data not shown).

As expected, no relation was found between plasma ddI and intracellular ddA-TP. This result can be explained by the complex intracellular anabolism of ddI (the monophosphate of dideoxyinosine undergoes a chemical transformation to dideoxyadenosine monophosphate) and/or the suggested longer intracellular

half-life of elimination for ddA-TP compared with the nucleoside form (approximately 30 h and 2 h, respectively), which leads to a difference in the pharmacokinetic profile between the plasma and intracellular forms.

The relationship between intracellular d4T-TP and plasma d4T is a new finding. Our result suggests that d4T phosphorylation is not a saturable process in the observed d4T plasma concentration range, despite the rate-limited formation of d4T monophosphate noted in vitro [3]. However, unlike zidovudine [4], no negative feedback by d4T anabolites on the kinase responsible for d4T intracellular anabolism had been previously demonstrated. The comparable correlation found with the whole sample ( $n = 26$ ) and for only the samples collected 2–4 h after d4T dosing ( $n = 16$ ) suggests that concentrations of the plasma nucleoside form d4T and the intracellular form d4T-TP follow a similar trend in time. This relationship between d4T and d4T-TP indicates that measurement of plasma d4T concentration could itself be clinically useful; however, this must be confirmed with non-responder patients since cellular factors can be partly responsible for drug inefficiency. No similar relation was found for ddI and ddA-TP and, therefore, ddA-TP determination appears necessary in any situation.

In the present study, a weak correlation was observed between ddA-TP and d4T-TP concentrations with samples collected 2–4 h after the last d4T dose and 9–19 h after the last ddI dose ( $n = 14$ ). This was not noted with the whole group of samples from the 26 compliant patients. Choosing a short time range limited concentration changes caused by anabolism and catabolism of the phosphorylated anabolites, which differ for the two drugs. However, this correlation is quite surprising, since d4T and ddI are intracellularly phosphorylated by two different pathways and no effect of ddI on d4T phosphorylation, or inversely, was observed [24,25]. Such a correlation has been described previously between the triphosphate anabolites of zidovudine and lamivudine in patients treated with a combination of the two NRTI [13]. Our explanation is that kinases involved in several NRTI phosphorylations are down- or upregulated and that there may be patients in whom phosphorylation is low or high, thus producing low or high concentrations of triphosphorylated anabolites, whatever the NRTI administered. This point has been discussed elsewhere [26]. However, this assertion could be formally proved by monitoring NRTI-TP concentrations in a large number of patients taking all the combinations of NRTI that are known not to interact and by seeking such correlations.

In conclusion, this is the first time that data have been obtained on the intracellular pharmacokinetics of the

active anabolites of d4T and ddI, d4T-TP and ddA-TP, the relation between plasma drug and active forms, and interpatient variability in naive responder patients.

The compatibility of the LC-MS/MS method with clinical studies has allowed such parameters to be examined. Clearly, other similar studies with more patients are needed to extend our understanding of the relationships between the intracellular pharmacokinetics of the active forms, immunological or virological parameters and the toxicity of these drugs (lipodystrophies, mitochondrial toxicity, etc.). Additionally, the relationship between plasma d4T and intracellular d4T-TP must be confirmed in non-responder patients. Since we observed that patients could be low or high metabolizers of NRTI, the final aim is to optimize NRTI therapy using quantification of active intracellular NRTI triphosphates, with the possibility that a poor response to NRTI could be related to a low intracellular concentration and toxicity related to over high concentrations.

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