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Evidence and Possible Consequences of the Phosphorylation of Nucleoside Reverse Transcriptase Inhibitors in Human Red Blood Cells[▽]

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The intracellular metabolism of nucleoside reverse transcriptase inhibitors (NRTI) in mononuclear cells has been thoroughly studied, but that in red blood cells (RBC) has been disregarded. However, the phosphorylation of other analogous nucleosides (in particular, ribavirin) has been described previously. In this study, we investigated for the first time the phosphorylation of NRTI in human RBC. The presence of intracellular zidovudine (AZT) monophosphate, AZT triphosphate, lamivudine (3TC) triphosphate, and tenofovir (TFV) diphosphate, as well as endogenous dATP, dGTP, and dTTP, in RBC collected from human immunodeficiency virus-infected patients was examined. We observed evidence of a selective phosphorylation of 3TC, TFV, and endogenous purine deoxynucleosides to generate their triphosphate moieties. Conversely, no trace of AZT phosphate metabolites was found, and only faint dTTP signals were visible. A comparison of intracellular TFV diphosphate and 3TC triphosphate levels in RBC and peripheral blood mononuclear cells (PBMC) further highlighted the specificity of NRTI metabolism in each cell type. These findings raise the issue of RBC involvement in drug-drug interaction, drug pharmacokinetics, and drug-induced toxicity. Moreover, the typical preparation of PBMC samples by gradient density centrifugation does not prevent their contamination with RBC. We demonstrated that the presence of RBC within PBMC hampers an accurate determination of intracellular TFV diphosphate and dATP levels in clinical PBMC samples. Thus, we recommend removing RBC during PBMC preparation by using an ammonium chloride solution to enhance both the accuracy and the precision of intracellular drug monitoring.

Nucleoside reverse transcriptase inhibitors (NRTI) constitute a peculiar category of antiretroviral drugs because they undergo multistep intracellular activation to exert the antiviral activity through the corresponding triphosphate, NRTI-TP. In fact, both the NRTI antiretroviral effect (13, 14, 18, 34) and mitochondrial toxicity (12) are related to the intracellular metabolism of NRTI. To date, intracellular pharmacological studies of patients have been focused on peripheral blood mononuclear cells (PBMC). These cells are the primary targets of human immunodeficiency virus (HIV) and are readily available from a single blood sample of around 7 ml. Concentrations of NRTI-TP in PBMC (and/or the ratio of the level of NRTI-TP to that of the deoxynucleoside triphosphate [dNTP], the corresponding natural triphosphate) (18) are now regarded as relevant to investigations of new NRTI (24), drug-drug interaction studies (9, 15, 17, 22, 27), and prescription strategies (33, 35). On the other hand, few studies (30) have addressed the role of NRTI phosphate concentrations in drug toxicity or in the persistence of virus in protected areas. Such investigations should be performed with different cell types (e.g., red blood cells [RBC], lymphocyte node mononuclear cells, adipocytes, and muscle cells), which are often difficult to collect from patients in clinical practice. Among these cell types, RBC are of interest since they could contain NRTI and the corresponding phosphorylated metabolites and can be easily collected from patients.

Indeed, despite previous studies of natural nucleoside metabolism in RBC (10, 32), little is known about intracellular deoxynucleotide triphosphates except their low concentrations (e.g., that of dATP is <0.1 pmol/10^6 normal RBC [7]). Note that RBC are known for their inability to synthesize purine nucleotides in the de novo pathway (10), which enables a unique nucleotide equilibrium to develop in RBC.

On the other hand, the metabolism of various analogous nucleosides in RBC has been studied previously. In particular, ribavirin (RBV), an anti-hepatitis C virus drug, is phosphorylated in RBC into its monophosphate, diphosphate, and triphosphate derivatives (RBV-MP, RBV-DP, and RBV-TP, respectively) (20, 36), which accumulate in high levels, leading to hemolytic anemia (19). The RBV-MP/RBV-DP and RBV-MP/RBV-TP ratios in RBC are significantly different from those in lymphoblasts or fibroblasts. The half-lives of these drug forms in RBC are also longer than those in other cell types. Similarly, tiazofurin (26) and 4-pyridone-3-carboxamide-1-β-D-ribonucleoside (29) are phosphorylated in RBC. These observations suggest that NRTI may be metabolized (i.e., phosphorylated) in RBC of patients undergoing NRTI therapy.

In addition, NRTI metabolism in RBC may hamper the measurement of NRTI-TP in PBMC. Indeed, various and unpredictable amounts of RBC remain in the PBMC samples despite the theoretical isolation of PBMC from the other blood components. This contamination, which is readily visible as it colors the PBMC pellet, is highly dependent on the characteristics of the blood donor and the experimental conditions of

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isolation. Hence, if endogenous and NRTI-TP nucleotides are present in RBC, PBMC contaminated with RBC will contain an enlarged pool of nucleotides, leading to a possible overestimation of both NRTI-TP and dNTP levels, an issue that needs to be evaluated.

In this paper, we address for the first time the phosphorylation of several NRTI, such as zidovudine (AZT), lamivudine (3TC), and tenofovir (TFV) disoproxil fumarate (TDF), in RBC. The pharmacological consequences of this metabolism regarding drug-drug interaction are examined. In addition, the possible bioanalytical consequences of deoxynucleoside metabolism in RBC for the determination of both NRTI-TP and dATP levels in PBMC samples contaminated with RBC and the improvement of PBMC preparation procedures are investigated.

MATERIALS AND METHODS

Materials and chemicals. 3TC, tenofovir, and NRTI phosphorylated anabolites (3TC triphosphate [3TC-TP], AZT monophosphate [AZT-MP], and AZT triphosphate [AZT-TP]) were obtained from Moravek Biochemicals (Brea, CA). 2-Chloro-ATP (Cl-ATP), AZT, dATP, dGTP, dTTP, 5-bromo-2′-deoxyuridine 5′-monophosphate (BrdU-MP), Ficoll–Histopaque-1077, formic acid (ammonium salt), NaCl, $NaHCO_3$, and 1,5-dimethylhexylamine were from Sigma-Aldrich. NH_4Cl was from SDS (Peypin, France). Ultrapure water was obtained by using a Milli-Q Plus 185 purifier (Millipore, France). Analytical acetonitrile was from Sigma-Aldrich (Darmstadt, Germany). Nitrogen and argon (both High Purity 45) were from Messer (Mitry, France). Human blood from healthy subjects was obtained from the French Blood Transfusion Service (Rungis, France).

Patients. Five to seven milliliters of venous blood was collected from HIV-infected patients within the framework of normal laboratory monitoring (Internal Medicine Unit, University Hospital of Bicêtre, Kremlin-Bicêtre, France; C. Goujard, director). All patients were receiving antiretroviral therapy that included TDF, 3TC, and AZT, either alone or in combination.

RBC lysis solution. A hypotonic ammonium chloride solution was adapted from that described by Elliott et al. (11) with minor modifications. NH_4Cl (3.5 g) and $NaHCO_3$ (0.036 g) were dissolved in exactly 500 ml of distilled water. The RBC lysis solution was stored at −4°C before use.

Stock and diluted solutions. Stock solutions of 3TC, AZT, and TFV were prepared by precisely weighing out 1 mg of powder, which was dissolved in 1 ml of ultrapure water and stored at −20°C. Stock solutions of 3TC-TP, AZT-MP, AZT-TP, TFV diphosphate (TFV-DP), dATP, dGTP, dTTP, BrdU-MP, and Cl-ATP were prepared in ultrapure water and stored at around −20°C (final concentrations, ~1 μg/ml for all but 3TC-TP, which had a final concentration of ~100 μg/ml). Diluted solutions for calibration standards and quality controls (QC) were prepared by the serial dilution of stock solutions in ultrapure water and were stored for up to 2 months at −80°C before use.

Preparation of blank, standard, and quality control samples. The experiments described below required blank PBMC and RBC samples (for the preparation of standard and quality control samples), which were isolated from 500 ml of blood (or plateletpheresis residues) by using standard density gradient centrifugation (usually 25 ml of blood over 15 ml of Histopaque-1077). After centrifugation (1,500 × *g*) at room temperature, PBMC were collected from a diffuse layer just above the gel and pooled in washing tubes. RBC were collected by pipetting below the gel. Both cell suspensions were washed three times in cold 0.9% NaCl solution (stored at 4°C) as previously described (3, 21). Cells were evenly distributed into microtubes at approximately 10×10^6 cells/tube. The supernatant was eliminated, and samples were immediately stored at −80°C. When required, before storage, blank PBMC pellets were artificially contaminated with known amounts of RBC (0, 50, 100, 150, and 200%) isolated from the same blood.

Preparation of incubated PBMC artificially contaminated with RBC. In vitro-incubated samples were prepared by spiking whole blood from healthy subjects (500 ml) with 5 μM 3TC–5 μM TFV and incubating for 24 h at 37°C. PBMC were prepared as described above for the blank samples. Before storage, PBMC pellets were artificially contaminated with known amounts of RBC (0, 50, 100, 150, and 200%) isolated from the same blood after incubation.

RBC lysis of PBMC samples. When required, RBC present within PBMC samples were removed by using an ammonium chloride solution (described above). Typically, cells were suspended in 2 ml of RBC lysis solution and left to stand for 2 to 3 min. Tubes were filled to 10 ml with cold NaCl solution (stored at 4°C) and immediately centrifuged for 5 min at 300 × *g* at 4°C. The supernatant was discarded, and the remaining cells were washed in cold NaCl solution before freezing.

PBMC sample treatment. Calibration standard and quality control samples were prepared from blank frozen PBMC samples spiked with 20 μl of the compound of interest in a solution at an adjusted concentration. The calibration ranges of TFV-DP, AZT-MP, AZT-TP, 3TC-TP, dTTP, and dATP concentrations were 95 to 4,900, 180 to 25,000, and 150 to 5,000 fmol/10^6 cells and 1.0 to 300, 0.3 to 25, and 0.3 to 25 pmol/10^6 cells, respectively. All samples (including patient and incubated PBMC samples) were then spiked with internal standards (i.e., Cl-ATP and BrdU-MP at 2.4 and 2.1 ng, respectively).

Mechanical lysis and nucleotide extraction from frozen PBMC pellets were achieved by adding 1 ml of 0.05 M Tris-HCl (pH 7.4)–methanol at a ratio of 3:7 to the cold pellet. Lysates were centrifuged (18,000 × *g* for 30 min at 4°C). Supernatants were collected, dried down at 37°C with a TurboVap (Zymark, France), and reconstituted with 150 μl of H_2O. Forty microliters of the final solution was injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) apparatus. The remaining solution was stored frozen (−80°C) until analysis, if required.

RBC sample treatment. Calibration standard and quality control RBC samples were prepared as described above for PBMC. The calibration ranges of TFV-DP, AZT-MP, AZT-TP, 3TC-TP, dTTP, and dATP concentrations were 95 to 4,900, 180 to 25,000, and 300 to 5,000 fmol/10^6 cells and 0.5 to 300, 0.3 to 25, and 0.3 to 24.7 pmol/10^6 cells, respectively.

Mechanical lysis and nucleotide extraction from frozen RBC pellets were first achieved by adding 0.5 ml of 0.05 M Tris-HCl (pH 7.4)–methanol at a ratio of 3:7 to the cold pellet. An additional 0.5 ml of methanol was added to the lysate to improve protein precipitation. Lysates were left at 4°C for 10 min before centrifugation (18,000 × *g* for 30 min at 4°C). Colorless supernatants were collected, dried down at 37°C with a TurboVap (Zymark, France), and reconstituted with 150 μl of H_2O. A 40-μl fraction of the final solution was injected into the LC-MS/MS apparatus. The remaining solution was stored frozen (−80°C) until analysis, if required.

Cell counting. Cell counting was performed by using two different methodologies. RBC were counted and RBC/PBMC ratios were determined before samples were frozen. A 20-μl aliquot of the cell suspension was diluted 1/1,000 (or 1/100, depending on the concentration of cells in the suspension) with trypan blue and counted by using a Malassez cell. PBMC were counted after freezing and cell lysis by using a validated biochemical test as previously described (4).

Analytical methodology. The LC-MS/MS assay methods used for NRTI-TP and dNTP were identical for PBMC and RBC samples. The assay was an improved version of previously described assays (8, 16, 22, 23). The liquid chromatography part of the assay was modified to allow the analysis to be run in 10.5 min. Briefly, the chromatographic separation was achieved by using an LC System 1100 (Agilent Technology, France) and a Supelcogel ODP 50 5-μm (50- by 2.1-mm) column (Sigma-Aldrich-Supelco) with the thermostat set at 30°C. Mobile phases A and B were 50:50 mixtures of 1,5-dimethylhexylamine (20 mM) and formate buffer (6 mM, pH 5) in ultrapure water or acetonitrile (21) and were delivered at a flow rate of 0.3 ml/min, as follows: 70% A from 0 to 0.6 min, 70 to 35% A from 0.6 to 4 min, 35 to 0% A from 4 to 4.3 min, 0% A for 0.9 min, 0 to 70% A from 5.2 to 5.5 min, and a final equilibration at 70% A for 4 min. At both the beginning (0 to 1 min) and the end (7 to 10.5 min) of the analysis, H_2O-acetonitrile (50/50, vol/vol) instead of the mobile phase was introduced at 0.3 ml/min by using an additional LC pump to wash the source. A triple quadrupole tandem mass spectrometer (a TSQ Quantum Ultra with an electrospray ionization source from Thermo-Electron) and treatment software (Xcalibur 1.4 and LCquan 2.0) were used for monitoring via data acquisition.

Usually, each sample underwent two complementary analyses, one using the electrospray-negative mode (for AZT-MP, AZT-TP, and dTTP quantification) and the other using the electrospray-positive mode (for 3TC-TP, TFV-DP, and dATP quantification, as well as dGTP monitoring). Both assays were fully validated for RBC samples with this mass spectrometer. Partial validation for PBMC samples, especially regarding the lower limit of quantification (LLOQ), was performed. Fragmentation was achieved with argon (collision gas pressure, 1.5 millitorrs).

In the electrospray-negative mode, the new parameters optimized for the ionization and detection of all compounds included a 3.5-kV spray voltage and a 350°C capillary temperature. The values of the tube lens offset were −76, −85, −85, −78, and −75 V for AZT-MP, AZT-TP, dTTP, Cl-ATP, and BrdU-MP, respectively. The ion transitions (collision energy) monitored were 346/177 (18 V), 506/380 (20 V), 481/159 (35 V), 540/159 (35 V), and 385/189 (20 V) for AZT-MP, AZT-TP, dTTP, Cl-ATP, and BrdU-MP, respectively.

In the electrospray-positive mode, the new parameters optimized for the

TABLE 1. Quantification of AZT-MP, AZT-TP, 3TC-TP, and TFV-DP in PBMC and RBC samples from HIV-infected patients[a]

Patient	NRTI treatment	Time (h) postdose	Concn (pmol/10^6 cells) of 3TC-TP in:		Ratio of 3TC-TP concn in RBC and PBMC	Concn (fmol/10^6 cells) of TFV-DP in:		Ratio of TFV-DP concn in RBC and PBMC	Concn (fmol/10^6 cells) of AZT-MP in:		Concn (fmol/10^6 cells) of AZT-TP in:	
			RBC	PBMC		RBC	PBMC		RBC	PBMC	RBC	PBMC
1	AZT-3TC	9.0	0.04	8.9	0.005				BLOQ	35.2	BLOQ	27.8
2	TDF	11.9				143	131	1.1				
3	3TC	NR	0.04	11.1	0.004							
4	3TC	0.3	0.09	10.5	0.009							
5	TDF	13.3				165	148	1.1				
6	TDF	0.8				443	226	2.0				
7	AZT-3TC	0.3	0.06	10.3	0.006				BLOQ	55.8	BLOQ	BLOQ
8	AZT-3TC	14.7	0.13	11.3	0.012				BLOQ	BLOQ	BLOQ	BLOQ
9	AZT-3TC	11.5	BLOQ	9.6					BLOQ	BLOQ	BLOQ	57.1
10	TDF	14.3				73.3	95.5	0.8				
11	TDF	13.5				245	202	1.2				
12	AZT-3TC	16.0	BLOQ	5.4					BLOQ	BLOQ	BLOQ	BLOQ
Mean					0.007			1.2				
CV (%)					47			36				

[a] CV, coefficient of variation; NR, not recorded; BLOQ, below the limit of quantification.

ionization and detection of all compounds included a 4.4-kV spray voltage and a 350°C capillary temperature. The values of the tube lens offset were 237, 165, 186, 185, and 192 V for 3TC-TP, TFV-DP, dATP, dGTP, and Cl-ATP, respectively. The ion transitions monitored (collision energy) were 470/112 (21 V), 448/176 (43 V), 492/159 (35 V), 508/152 (34 V), and 542/170 (35 V) for 3TC-TP, TFV-DP, dATP, dGTP, and Cl-ATP, respectively.

Results obtained from the LC-MS/MS analysis were the ratio of each analyte's peak area to that of the corresponding internal standard (Cl-ATP for all triphosphates and BrdU-MP for AZT-MP). For TFV-DP, 3TC-TP, AZT-MP, AZT-TP, dATP, and dTTP, a calibration curve was plotted by using at least six calibration standards. The best fits were obtained with a linear regression analysis (weighted $1/x$ for TFV-DP, 3TC-TP, and AZT-MP and weighted $1/x^2$ for AZT-TP). Since dATP and dTTP are endogenous compounds naturally present in blank PBMC and RBC samples, the endogenous dNTP quantities were taken into account by subtracting the blank value from the calibration curve as previously described (16).

For PBMC samples, intra- and interday precision and accuracy levels (data not shown) were as given in prior publications (8, 16, 23). For RBC samples, intra- and interbatch precision levels were calculated at four concentrations (the LLOQ and concentrations corresponding to low QC, medium QC, and high QC) covering the range of the calibration. For positive electrospray ionization, interrun precision (and accuracy) levels at the LLOQ were 16.2% (120%), 14.9% (111%), and 22.6% (80.1%) for TFV-DP, 3TC-TP, and dATP, respectively. Overall, the mean accuracy ranged between 93.1 and 107% for TFV-DP, 103 and 108% for 3TC-TP, and 101 and 108% for dATP. For negative electrospray ionization, interrun precision (and accuracy) levels at the LLOQ were 17.2% (111%), 11.9% (118%), and 19.6% (120%) for AZT-MP, AZT-TP, and dTTP, respectively. Overall, the mean accuracy ranged between 91.1 and 108% for AZT-MP, 88.4 and 106% for AZT-TP, and 100 and 111% for dTTP.

Data analysis. All results expressed as femtomoles or picomoles per sample were divided by the number of cells to obtain the concentration in femtomoles or picomoles per 10^6 cells. Results are presented either as mean ratios ± standard deviations or as mean evaluated concentrations ± standard deviations. Different groups of samples were compared using Student's t test and one-way analysis of variance (ANOVA) with GraphPad Prism 3.02 (GraphPad Software). A value of P of <0.05 was considered statistically significant.

RESULTS

Presence of NRTI-TP and dNTP in RBC. In order to investigate and confirm the presence of nucleotides in RBC, NRTI-TP (TFV-DP, 3TC-TP, AZT-TP, and AZT-MP) and dNTP (dATP, dGTP, and dTTP) in RBC samples from healthy donors and NRTI-treated patients were monitored directly (Table 1). PBMC samples collected from the same patients were analyzed for comparisons.

As expected, no NRTI monophosphate or NRTI-TP signals in RBC from healthy volunteers were observed. 3TC-TP or TFV-DP was detected specifically when the antiretroviral therapy of HIV-infected donors included 3TC or TFV, respectively. We observed similar intracellular concentrations of TFV-DP in RBC and PBMC samples (range, 73 to 443 and 96 to 226 fmol/10^6cells, respectively; mean RBC/PBMC ratio, 1.2). Intracellular levels of 3TC-TP in RBC ranged between 0.04 and 0.13 pmol/10^6cells, whereas concentrations in PBMC ranged between 5.4 and 11.3 pmol/10^6 cells (mean RBC/PBMC ratio, 0.007). We observed no traces of AZT-MP or AZT-TP in RBC from AZT-treated patients.

Intracellular dGTP levels in RBC samples were not quantified, but the presence of dGTP was nevertheless observed. Moreover, dATP signals were detected on chromatograms from all RBC samples but were always close to or below the LLOQ. Conversely, dTTP signals were either faint or missing on chromatograms from RBC samples.

Effect of RBC contamination of patient PBMC samples on TFV-DP measurements. From among suspensions of PBMC from HIV-infected patients treated with TDF, we selected three samples (those from patients A1 to A3) (Table 2) visibly contaminated with RBC. The percentage of contamination of each sample was evaluated by using a Malassez cell. Then one half of each sample was frozen, whereas the other half was submitted to an additional RBC lysis step and NaCl washing before freezing. TFV-DP in samples was quantified by using the LC-MS/MS assay previously described. A comparison of RBC-contaminated PBMC samples with the corresponding RBC-lysed PBMC samples showed important differences in TFV-DP concentrations (up to sevenfold).

Matrix effect: effect of RBC contamination of blank PBMC samples on mass spectrometry nucleotide signals. In order to examine a potential RBC-related matrix effect on the quantification of nucleotides in PBMC, we artificially contaminated

TABLE 2. Quantification of TFV-DP in RBC-contaminated PBMC samples (prepared with or without the removal of RBC) from HIV-infected patients

Patient	NRTI treatment	Time (h) postdose	% Contamination with RBC[a]	Concn (fmol/sample) of TFV-DP in:		Ratio of TFV-DP concn in nonlysed sample and lysed sample
				Nonlysed sample	RBC-lysed sample	
A1	TFV	2.0	>200	849.6	395.4	2.15
A2	TFV	9.5	>200	1,287	182.5	7.05
A3	TFV	2.0	50	302.6	376.6	0.80

[a] To determine the level of contamination, the RBC/PBMC ratio was calculated and expressed as a percentage.

blank PBMC samples with known amounts of RBC. During the analytical process, sample extracts were spiked with TFV-DP and 3TC-TP (at the low-QC concentration), whose presence was monitored by using the LC-MS/MS assay previously described. Chromatographic peak area ratios were identical for the two compounds: only small variations (±15%) associated with the different levels of RBC contamination were observed. None of these findings were significant (Fig. 1) ($P > 0.05$; ANOVA).

Intracellular dATP was simultaneously monitored. We observed a significant increase of the dATP peak area ratio in proportion to the presence of RBC in the PBMC sample (Fig. 1) (P, <0.0001 by ANOVA; linear regression, $r^2 = 0.8497$).

Effect of RBC contamination of in vitro-incubated PBMC samples on determination of 3TC-TP, TFV-DP, and dATP levels. PBMC samples incubated in vitro with 5 μM 3TC–5 μM TFV and artificially contaminated with RBC were prepared either by the standard procedure or by an improved procedure including a specific RBC lysis step performed instead of the second washing in NaCl solution. While different levels of RBC contamination of PBMC samples were at first easily identified by the sample colors, they became highly unpredictable after the RBC lysis step because all the samples turned colorless. The results of TFV-DP, 3TC-TP, and dATP monitoring with the LC-MS/MS assay in the positive ionization mode are shown in Fig. 2. When samples were processed without RBC lysis, the measurements of TFV-DP and dATP were significantly influenced by the presence of RBC ($P < 0.01$; ANOVA). In contrast, no effect on 3TC-TP signals was noted ($P > 0.05$; ANOVA). Conversely, when sample processing included an RBC lysis step, only small and nonsignificant signal variations (±15%) between the samples were observed ($P > 0.05$; ANOVA).

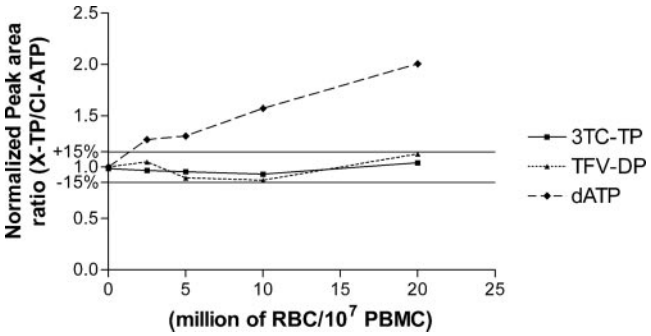

FIG. 1. RBC-related matrix effect. Levels of 3TC-TP, TFV-DP, and dATP were monitored in extracts from blank samples of 10^7 PBMC artificially contaminated with increasing amounts of RBC (0 to 20×10^6 RBC) and spiked with 3TC-TP and TFV-DP (at the low-QC concentration; $n = 5$). X-TP, indicated phosphate.

DISCUSSION

In order to assess the in vivo NRTI phosphorylation in human RBC, we analyzed RBC samples from HIV-infected patients for the presence of NRTI monophosphates and NRTI-TP. For this purpose, we adapted an LC-MS/MS assay previously developed for the measurement of NRTI phosphate metabolites in PBMC to be used with RBC samples. First, we demonstrated the presence of 3TC-TP and TFV-DP in RBC samples from HIV-infected patients (Table 1). Conversely, no AZT phosphate metabolites in RBC were evident. The monitoring of endogenous deoxynucleotides in RBC offered similar results: significant traces of dATP and dGTP on chromatograms were observed, whereas dTTP signals were barely detectable or missing. Then we demonstrated the presence, in RBC, of an active phosphorylation pathway for deoxynucleosides (in particular, 3TC and TFV) except thymidine-based ones. Indeed, only faint traces of dTTP on some chromatograms were observed, and no AZT phosphate metabolites in RBC samples from AZT-treated patients were detected. Considering that the sampling time of these samples was near the new dosing (concentration close to C *trough*), we cannot conclude whether phosphorylation activity towards thymidine-based deoxynucleosides is either too low to be evident or lacking.

In order to compare the patterns of phosphorylation of NRTI in RBC and PBMC, NRTI-TP levels in samples of both cell types collected from the same individuals were determined. We calculated slightly higher concentrations of TFV-DP in RBC than in PBMC samples when the concentrations were expressed as femtomoles per 10^6 cells (mean RBC/PBMC ratio, 1.2). However, when we considered the size of each type of cell (RBC, around 0.096 pl, and PBMC, around 0.187 to 0.218 pl [6, 28]), the differences appeared to be even greater, with intracellular nucleotide concentrations twice as high in RBC as in PBMC. Conversely, 3TC-TP levels in RBC were very low compared to those in PBMC (mean RBC/PBMC ratio, 0.007). Concentrations of AZT-MP and AZT-TP in PBMC samples were often too low to be quantified. These results can be explained by the fact that the concentrations of all samples were obtained close to C *trough* and by the low number of PBMC per sample (range, 1.9×10^6 to 6.2×10^6). Nevertheless, AZT phosphates are always missing in RBC.

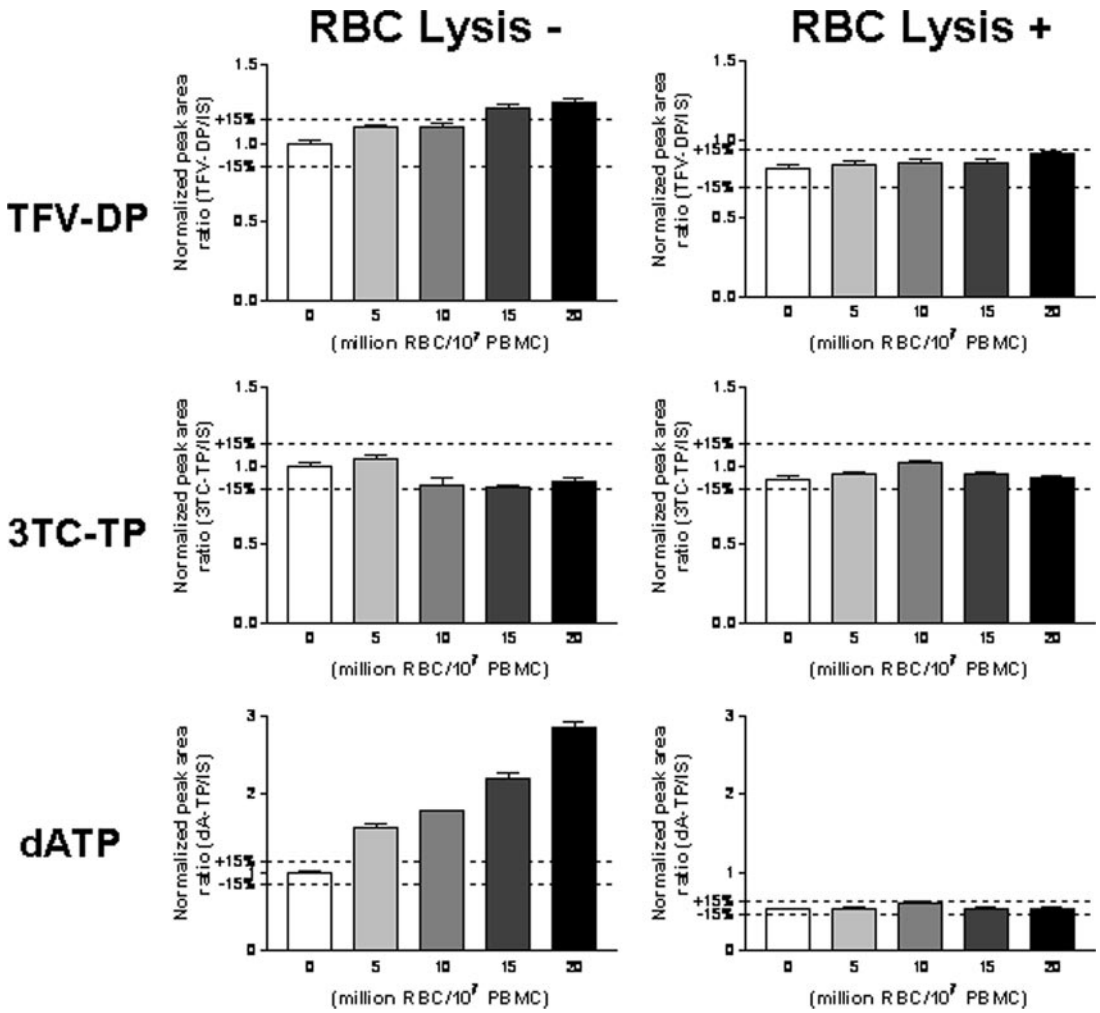

FIG. 2. Influence of RBC lysis on measurements of TFV-DP, 3TC-TP, and dATP in samples of 10^7 PBMC obtained from whole blood incubated with 5 μM TFV–5 μM 3TC for 24 h and artificially contaminated with RBC isolated from the same blood after incubation (0 to 20 × 10^6 RBC; $n = 4$). RBC lysis−, no RBC lysis; RBC lysis+, samples were subjected to RBC lysis.

This observation suggests reduced AZT phosphorylation in RBC, which will have to be confirmed in further studies.

We have demonstrated for the first time that NRTI undergo selective phosphorylation in RBC. Data suggest than only nonthymidine-based nucleosides are efficiently phosphorylated in this cell type and reveal different magnitudes of metabolism in RBC and PBMC. These discrepancies may be explained by considering the inability of RBC to synthesize purine nucleotides in the de novo pathway (10) and/or other differences in metabolic activation and/or degradation pathways.

NRTI phosphorylation in RBC needs to be more thoroughly investigated because of the potential consequences regarding drug pharmacokinetics and drug-drug interaction. First, such information may help in building a compartmental model for NRTI pharmacokinetics. Second, the phosphorylation of TFV in RBC may contribute, to some extent, to the dideoxyinosine (ddI)-TDF interaction. Indeed, the molecular mechanism of ddI interaction with TDF is explained by the inhibition of ddI breakdown into hypoxanthine by purine nucleoside phosphorylase (PNP). In fact, Ray et al. (25) established that the coadministration of ddI and TFV decreased the amount of intracellular ddI breakdown products in CEM cells (human leukemic CCRF-CEM T lymphoblasts) while it increased ddI concentrations. The authors suggest a direct inhibition of PNP activity by TFV phosphorylated metabolites in lymphoblasts. However, PNP is a ubiquitous enzyme whose highest levels of activity are found in kidney cells, PBMC, RBC, and granulocytes (5). Because of the large portion of blood volume taken up by RBC, coupled with their high PNP levels, RBC have previously been proposed as a major site for ddI clearance (1). In that case, the large increase in the plasma ddI concentration observed with TDF coadministration cannot be explained only by the inhibition of the ddI breakdown within mononuclear cells. Nuclear cells does not explain the large increase in the plasma ddI concentration observed with TDF coadministration (increase of ddI plasma ranging from area under the curve 48 to 64%). In demonstrating high levels of TFV-DP in RBC, we shed new light on PNP-related interaction mechanisms. The inhibition of ddI clearance by TFV phosphorylated metabolites may occur in both RBC and mononuclear cells, explaining

the major increase in the plasma ddI concentration observed in clinical practice.

Moreover, it is common knowledge that PBMC suspensions prepared by standard gradient density procedures include various, unpredictable, but readily visible amounts of RBC. Despite the training of clinical staff, multisite examinations of sample preparation have shown a wide range of contamination levels (in our experience, 0 to 200%). This phenomenon can be explained by variations in instrumentation and laboratory practices (e.g., pipette overlaying of blood on Ficoll-Histopaque and an awareness of problems related to contaminating RBC, etc.), which may enhance the mean contamination rate. The potential influence of RBC on the intracellular analysis of contaminated PBMC samples cannot be ignored but has never been investigated.

In order to allow a comprehensive study of the consequences of contamination on intracellular analyses of RBC-contaminated PBMC samples, we prepared in vitro-incubated PBMC. Firstly, the presence of RBC within PBMC may modify the chemical composition of the PBMC extracts and thus potentially cause ion suppression or interference in LC-MS/MS, leading to a so-called matrix effect, which may ruin both the accuracy and the reproducibility of the assay results. Such an effect with 3TC-TP and TFV-DP was investigated in the present study but was not significant within the tested contamination range (RBC/PBMC ratio, 0 to 2) (Fig. 1). However, in these samples, an increase of dATP signals proportional to the level of RBC contamination was observed. Considering the close chemical structures, tandem mass spectrometry fragmentation patterns, and retention times of dATP and other nucleotides, a specific dATP matrix effect is unlikely. But the presence of dATP in RBC (as already evidenced in patient samples) may induce a significant increase of dATP depending on the degree of RBC contamination.

Secondly, in lysing RBC-contaminated PBMC samples, intracellular metabolites from RBC and PBMC are mixed in a single extract, leading to a potentially significant overestimation of NRTI-TP and dNTP concentrations (expressed as moles per 10^6 PBMC) in these samples. Proof of this concept was obtained when RBC-contaminated and uncontaminated PBMC samples from TDF-treated patients were compared (Table 2). Indeed, significantly higher TFV-DP concentrations in samples heavily contaminated with RBC were measured, establishing an RBC-related overestimation of TFV-DP levels. In the light of these results, some unexpectedly high intracellular concentrations of TFV-DP reported in previous pharmacological studies (15, 22) may be explained by the presence of a high level of RBC contamination of PBMC samples.

Such a phenomenon was then studied by using artificially contaminated PBMC samples from blood incubated in vitro with TFV-3TC (Fig. 2). On one hand, both dATP and TFV-DP measurements increased with the presence of RBC in PBMC samples, demonstrating the negative influence of RBC within PBMC samples. On the other hand, only small differences in 3TC-TP levels were observed (±15%, i.e., within the analytical variability). Indeed, considering the ratio of 3TC-TP concentrations in RBC and PBMC from 3TC-treated patients (RBC/PBMC ratio, 0.007) (Table 1), the RBC-related 3TC-TP increase is not significant in the tested contamination range.

We then demonstrated that the intracellular analysis of PBMC samples contaminated with RBC can lead to an overestimation of TFV-DP and dATP levels but that contamination does not disturb the determination of 3TC-TP concentrations. All the same, no influence of RBC on measurements of AZT-TP in PBMC is expected as the phosphorylation of AZT in RBC could not be indicated. Finally, as this RBC-associated overestimation is related strictly to the PBMC isolation procedure and the intracellular RBC metabolism, it is not specific to LC-MS/MS assays and should be considered with any analytical methodology.

In order to get rid of this RBC influence, the advantages of a specific RBC lysis step in the preparation of PBMC samples were examined in terms of signal variation and precision. RBC were eliminated by using an ammonium chloride solution, but there are other formulas (31) as well as commercial solutions which could be similarly used. The process is simple and fast (around 3 min plus centrifugation time) and does not affect PBMC viability (data not shown). Moreover, this lysis step can easily be performed in clinical units without slowing the PBMC preparation, as it can be used as a replacement for the first or second cell washing in the standard PBMC isolation procedure (2).

The efficiency of the RBC lysis of in vitro-incubated PBMC samples artificially contaminated with RBC was evaluated by subjecting the contaminated samples to an RBC removal step or leaving them untreated (Fig. 2). Whereas an RBC-related influence on measurements of TFV-DP and dATP in PBMC samples contaminated with RBC was evident, no significant changes in RBC-lysed samples were observed. For all the tested analytes, nucleotide concentrations in RBC-lysed PBMC samples were equal to (±15%; 3TC-TP) or lower than (TFV-DP and dATP) those in nonlysed samples. This difference, observed only for dATP and TFV-DP, is not surprising as it may be related only to the elimination of the basal RBC contamination of the PBMC suspension we used. Finally, the analytical variability of intracellular concentrations in PBMC samples was not significantly enhanced by the additional RBC lysis step. Overall, with the use of the improved preparation procedure for PBMC samples, nucleotide determination is no longer influenced by RBC contamination and is therefore more accurate.

Conclusions. Evidence of an active phosphorylation of nonthymidine-based NRTI in RBC has been presented in this paper. However, AZT phosphorylation could not be established. These new findings call for further studies for a full investigation of the metabolism of all NRTI in this cell type. Such data may help explain and/or predict the effects of pharmacokinetic profiles and toxicity, such as hematologic anemia (19) and drug-drug interaction.

In addition, we emphasized the negative influence of RBC contamination of PBMC on the determination of NRTI-TP or natural dNTP concentrations. This influence is a consequence of the presence of high levels of some of these nucleotides (in particular, TFV-DP and dATP) in RBC. Therefore, we recommend removing RBC during the PBMC preparation process. RBC lysis is fast and simple and can easily be performed at the clinical unit to eliminate RBC-related artifacts, which

potentially lower accuracy and precision. An acknowledgment of these key requirements is not restricted to NRTI monitoring but should enhance the quality of any drug monitoring associated with PBMC.

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