

Significant levels of intracellular stavudine triphosphate are found in HIV-infected zidovudine-treated patients

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Design and objective: It has been previously shown that zidovudine (ZDV) and its phosphorylated metabolites can be chemically reduced into the corresponding stavudine (d4T) forms in solution. The aim of this study was to search for intracellular d4T-triphosphate (TP) in patients receiving ZDV therapy as part of highly active antiretroviral therapy and to examine the ratio of concentrations of d4T-TP : ZDV-TP in these patients.

Methods: Seven ml of blood were sampled between 0.5 and 13.7 h after the last ZDV dosing in 31 patients. Peripheral blood mononuclear cells (PBMC) were separated using Vacutainer[®] CPT[™] tubes. Intracellular d4T-TP and ZDV-TP concentrations were determined by a newly developed high performance liquid chromatography/tandem mass spectrometry method.

Results: Intracellular d4T-TP was found in all ZDV-treated patients. d4T-TP concentrations ranged between 3 and 38.5 fmol/1 × 10⁶ cells and represented between 0.03 and 0.37 of the corresponding ZDV-TP concentrations. These d4T-TP concentrations are in the lower range of those measured in d4T-treated patients. The intracellular transformation of ZDV into d4T-TP was also observed during *in vitro* experiments in cells cultured in the presence of ZDV. d4T-TP was never detected in PBMC from patients treated with neither ZDV nor d4T.

Conclusion: Significant levels of d4T-TP can be measured in PBMC from patients receiving ZDV therapy. This observation sheds new light on the cross resistance observed between ZDV and d4T and indicates that, in patients treated with ZDV, d4T-TP could participate in the antiretroviral activity and/or toxicity of the drug.

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Introduction

Nucleoside analogue reverse transcriptase inhibitors (NRTI) belong to the first category of drugs that was shown to have antiretroviral activity against HIV. These NRTI must be transformed intracel-

lularly to their corresponding triphosphate moiety (NRTI-TP) in order to interfere with the reverse transcriptase activity of the virus working as competitive inhibitors and chain terminators. Both therapeutic efficacy and toxicity of NRTI are thought to be directly related to the intracellular concentration

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of NRTI-TP which are the actual active principle of the drug.

It has been established that the active anabolite of zidovudine (ZDV), the first compound to be used against HIV, and stavudine (d4T), another widely used NRTI for AIDS treatment, are ZDV-TP [1] and d4T-TP [2], respectively. The intracellular anabolism of these drugs has been established mainly by the means of *in vitro* experiments using cells (mainly cell lines) cultured in the presence of the radiolabelled NRTI. *In vivo* studies have confirmed the presence of ZDV-TP in ZDV-treated patients and in healthy volunteers [3,4].

In the present study, we demonstrate that the intracellular metabolism of ZDV leads also to significant levels of d4T-TP in peripheral blood mononuclear cells (PBMC) of HIV-infected patients.

Materials and methods

In vivo studies

The presence of d4T-TP in clinical samples was

checked in PBMC obtained from 31 HIV-infected patients treated with various antiretroviral combinations. These combinations as well as the corresponding number of patients are shown in Table 1. Blood samples (7 ml) were collected 0.5–13.75 h after ZDV dosing at the Internal Medicine Unit, Bicêtre Hospital, France within the framework of normal laboratory monitoring, and PBMC were isolated as described previously [5]. All subjects gave informed consent for participation in this study.

In vitro studies

NS-1 cells (non-secreting myeloma) were cultured for 2 days in the presence of: 0, 5, 20 and 50 μM ZDV. PBMC activated with phytohemagglutinin (1 $\mu\text{g}/\text{ml}$) were cultured for 3, 24 and 80 h in the presence of: 0, 1 or 20 μM ZDV.

NRTI-TP quantification

d4T-TP was quantified according to the previously described liquid chromatography tandem mass spectrometric (LC/MS/MS) method [5]. Quantification limit and detection limit were established at 61 and 20 fmol of d4T-TP per sample, respectively. ZDV-TP was determined using a similar analytical method coupled

Table 1. Intracellular zidovudine (ZDV) triphosphate (TP) and stavudine (d4T)-TP concentrations in peripheral blood mononuclear cells of patients treated with ZDV.

Patient	Associated NRTI	Time between ZDV dosing and blood sampling (h)	d4T-TP concentration (fmol/1 $\times 10^6$ cells)	ZDV-TP Concentration (fmol/1 $\times 10^6$ cells)	Ratio d4T-TP:ZDV-TP
1	3TC-ddl	6.2	BLQ	BLD	
2	3TC	12.5	BLQ	97.7	
3	ddl	1.5	BLQ	316	
4	ddC	3.2	BLQ	376	
5	3TC-ABC	10.0	BLQ	ND	ND
6	3TC-ABC	2.0	3.0	31.3	0.096
7	3TC	2.5	6.2	137	0.045
8	3TC	13.7	8.7	54.1	0.165
9	3TC-ABC	2.5	9.10	97.0	0.094
10	3TC	0.5	9.4	64.5	0.146
11	3TC	12.2	9.5	55.6	0.171
12	3TC	11.2	10.1	ND	ND
13	3TC	3.0	10.4	128	0.081
14	3TC	2.0	10.4	ND	ND
15	3TC	11.5	11.4	386	0.030
16	3TC-ABC	10.5	11.5	ND	ND
17	3TC	0.7	12.9	72.6	0.178
18	3TC	2.5	14.7	190	0.077
19	3TC	10.4	16.1	ND	ND
20	ddC	5.0	17.9	97.9	0.183
21	3TC	12.0	19.0	152	0.125
22	3TC	2.3	20.3	ND	ND
23	3TC	13.7	20.4	ND	ND
24	3TC	9.2	20.5	ND	ND
25	3TC	10.5	20.8	ND	ND
26	3TC	10	22.2	81.1	0.274
27	3TC	11.7	23.4	212	0.110
28	3TC	1.0	24.2	188	0.129
29	3TC	0.5	30.6	240	0.128
30	3TC-ABC	ND	35.7	ND	ND
31	3TC	13.5	38.5	105	0.367

NRTI, Nucleoside analogue reverse transcriptase inhibitor; 3TC, lamivudine; ABC, abacavir; ddC, zalcitabine; BLQ, below limit of quantification; BLD, below limit of detection; ND, not determined.

with immunoaffinity chromatography [6]. Quantification limit and detection limit were established at 93 and 31 fmol of ZDV-TP per sample, respectively.

Briefly, chromatography was achieved on a Supelcogel ODP-50 5 μm , 150 \times 2.1 mm (Supelco, St Quentin-Fallavier, France) maintained at 30°C. A gradient comprising dimethylhexylamine, as ion pairing reagent, ammonium formate pH approximately 11.5 mixed with acetonitrile was delivered at a flow rate of 0.3 ml/min. An HPLC system 1100 (Agilent Technology, Les Ulis, France) connected to an API 3000 tandem mass spectrometer with an electrospray source operating in the negative mode (Sciex, Applied Biosystem, France) was used. Fragmentation was achieved with nitrogen. The ion transitions monitored are as follows: 463/159, 506/159, and 540/159 for d4T-TP, ZDV-TP and 2-chloroadensine triphosphate (CIA-TP as internal standard), respectively. The retention times reached 10.5–11, 12–12.5 and around 11 min for d4T-TP, ZDV-TP and CIA-TP, respectively. For maximum sensitivity of NRTI-TP detection, the MS parameters were optimized for each compound.

Plasma d4T concentration was determined using a competitive enzyme immunoassay after solid-phase ex-

traction. d4T extraction was performed on Oasis cartridge (Waters). d4T was eluted with 1 ml methanol. After drying under nitrogen, d4T was diluted in 500 μl phosphate-BSA buffer [7]. Competitive enzyme immunoassay was performed using rabbit anti-d4T antibodies and d4T coupled to the enzyme acetylcholinesterase as described previously for the measurement of ZDV-MP [7].

Results

When analysing PBMC samples from ZDV-treated patients who received 300 mg ZDV twice daily, we made the observation that in all of them, a mass spectrometric signal corresponding to detectable d4T-TP levels (above the limit of detection) was found ($n = 31$; Table 1). In these samples, d4T-TP concentrations were above the limit of quantification of our assay (see legend of Table 1) for 26 out of 31 patients. As an illustration, MS/MS chromatograms for one of the ZDV-treated patients is shown in Fig. 1.

ZDV-TP concentrations were determined for 21 of the 31 ZDV-treated patients. The ratio d4T-TP : ZDV-TP

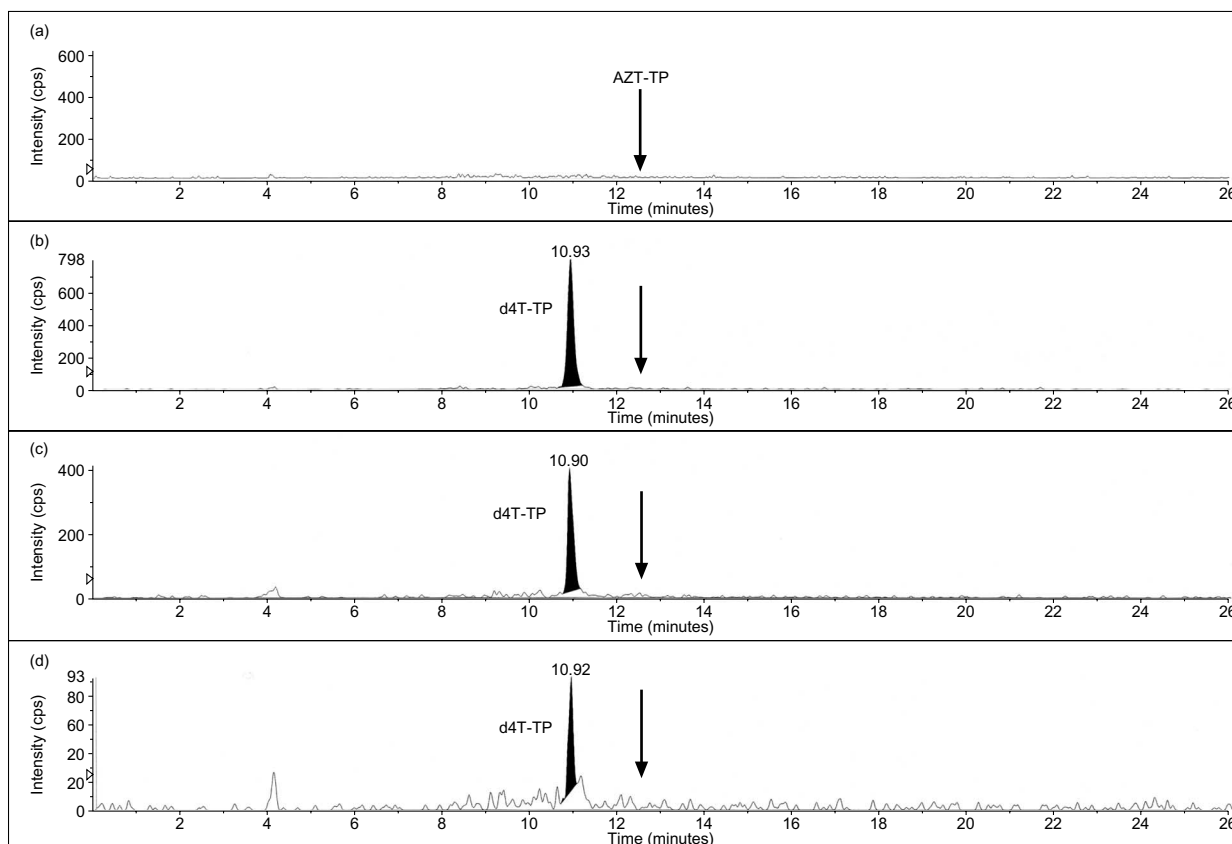


Fig. 1. LC/MS/MS chromatograms (monitoring of transition 463/159) in PBMC extracts. (a) Extract of PBMC from patient receiving neither ZDV nor d4T (control). (b) Extract of PBMC from a healthy volunteer spiked with a d4T-TP standard. (c) Extract of PBMC from a patient under d4T therapy, and (d) Extract of PBMC from a patient under ZDV therapy.

in PBMC ranged from 0.03 to 0.367 (median, 0.128; Table 1).

In contrast, no mass spectrometric signal corresponding to d4T-TP was observed in PBMC from patients for which therapy excluded both ZDV and d4T and who were treated with other combinations of NRTI ($n = 15$). Measurements performed on 20 plasma samples from patients taking ZDV, using a newly developed enzyme immunoassay (see methods), have shown that no quantifiable levels of d4T were present in the blood.

These *in vivo* observations are supported by *in vitro* experiments performed on cultured cells. When culturing either NS-1 cells or PBMC in the presence of ZDV, both ZDV-TP and d4T-TP were systematically found in the intracellular compartment (Table 2). In contrast, none of these NRTI-TP could be detected in control cells cultured in the absence of NRTI. In NS-1 cells, intracellular ZDV-TP and d4T-TP concentrations increased with ZDV concentration in the culture medium, but the ratio d4T-TP:ZDV-TP remained constant at 0.021 (coefficient of variation, 6%; $n = 3$; Table 3). In PBMC, the same dependence of intracellular NRTI-TP content on ZDV concentration in the culture medium was observed but a higher d4T-TP:ZDV-TP ratio was observed (0.06 versus 0.03) when extracellular ZDV increased from 1 to 20 μM (Table 2).

The assignment to d4T-TP of the signal detected in cells cultured in the presence of ZDV is supported both by the m/z ratio (characteristic of d4T) and by the identity between daughter ions of $m/z = 463$ obtained from samples coming either from ZDV-treated or d4T-TP spiked cells (data not shown).

Discussion

This report clearly shows that ZDV, a thymidine analogue known to be anabolized intracellularly into ZDV-TP (supposed to be the active form of the drug), is also intracellularly transformed both *in vitro* and *in vivo*, into d4T-TP, the active anabolite of d4T, another thymidine analogue structurally related to ZDV (Fig. 2). The validity of this observation is supported by the following arguments: (i) the analytical method used for this assessment is one of the most specific in the field of the clinical pharmacology, as it combines the selectivity of LC (retention time and peak shape) with the very high selectivity of MS/MS which operates on two analysing filters: the first quadripole selects only the compound with the mass/charge ratio of d4T-TP (463) and the second quadripole selects only the mass/charge ratio of the fragment pyrophosphate (159) originating from the fragmentation of the parent compound with a mass/charge ratio of 463; (ii) the daughter ion mass spectrum, which represents the fingerprint of a compound, exhibited the same three main fragments, thus unambiguously identifying the

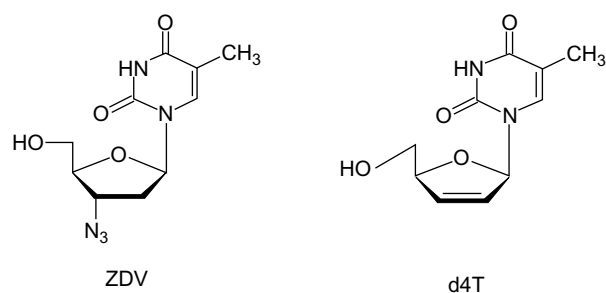


Fig. 2. Structure of ZDV and d4T.

Table 2. Intracellular zidovudine (ZDV) triphosphate (TP) and stavudine (d4T)-TP concentrations in NS-1 cells and peripheral blood mononuclear cells (PBMC) cultured in the presence of ZDV.

ZDV concentration in culture medium (μM)	Duration of incubation (h)	ZDV-TP (fmol/1 $\times 10^6$ cells)	d4T-TP (fmol/1 $\times 10^6$ cells)	Ratio d4T-TP:ZDV-TP
NS-1 cells				
0	48	BLD	BLD	–
5	48	13.1	271	0.0206
20	48	13.5	294	0.0217
50	48	66.5	1277	0.0192
PBMC				
0	3	BLD	BLD	
0	24	BLD	BLD	
0	80	BLD	BLD	
1	3	24.4	BLD	
1	24	218.1	6.0	0.027
1	80	333.9	12.1	0.036
20	3	82.3	BLD	
20	24	260.9	15.4	0.059
20	80	355.6	22.2	0.063

BLD, Below limit of detection.

detected product as authentic d4T-TP (data not shown); (iii) the chemical transformation of ZDV-TP into d4T-TP by a reducing treatment was previously shown [8]. The mechanism of the reduction would be abstraction of the 2'-proton followed by elimination of the reduced azido group; (iv) most of d4T-TP concentrations measured in ZDV-treated patients are clearly above the limit of quantification of the method and are thus well detected and quantified; (v) the link between d4T-TP levels measured in ZDV-treated patients and ZDV is supported by *in vitro* experiments showing that d4T-TP is produced in cells cultured in the presence of ZDV while d4T-TP was never detected in patients not receiving either ZDV or d4T but who were treated with other NRTI; (iv) we can exclude the possibility that d4T-TP was formed during the analytical process (cell preparation, cell lysis, chromatographic procedure, electrospray ionisation) because the spiking of PBMC from healthy volunteers, before the lysis of the cells, by ZDV or ZDV-TP never led to the production of a peak corresponding to d4T-TP (data not shown).

d4T-monophosphate (MP) and d4T-diphosphate (DP) were not detected either in patients treated with ZDV or during *in vitro* experiments. This is not surprising as neither d4T-MP nor d4T-DP were found in the PBMC of patients treated with d4T [9]. The absence of signal for d4T-MP and d4T-DP in patients taking d4T therapy does not confirm previous observations made during *in vitro* experiments with cell lines cultured in the presence of radiolabelled d4T [2]. We believe that this shows that the *in vivo* intracellular metabolism of NRTI in PBMC cannot be predicted from data generated *in vitro* in cell lines. In line with previous works (for a review see [10]), we suggest that in PBMC the limiting step in the d4T phosphorylation pathway is the transformation of d4T to d4T-MP, whereas d4T-MP and d4T-DP are good substrates for thymidilate kinase and nucleotide diphosphate kinase respectively [11]. Thus, except in cases of perturbed phosphorylation, no accumulation of mono- or diphosphorylated compounds of d4T occurs and concentrations of these anabolites are therefore negligible compared with those of d4T-TP.

In general, the differences observed between *in vitro* experiments (cultured cells) and *in vivo* measurements (patients) may explain why the intracellular production of d4T-TP from ZDV was not recorded before. In fact, it seems very likely that in cell lines very low levels of d4T-TP are produced compared with ZDV-TP as exemplified by our results with NS-1 cells (ratio 0.02) whereas higher ratios are observed in patients (up to 0.37). In addition, the chromatographic methods used for separating mono-, di- and triphosphate from the parent nucleoside were not adapted for the separation of ZDV-TP from d4T-TP, as the production of d4T-TP was not expected in these experiments.

Considering all of the data together, it is not possible to conclude on which species (nucleoside, mono-, di- and/or triphosphate) this unusual *in vivo* transformation of ZDV into d4T occurs. The transformation seems to be restricted to the intracellular compartment as no quantifiable levels of d4T were detected in the plasma of patients on ZDV therapy.

Intracellular d4T-TP concentrations measured in PBMC from ZDV-treated patients ranged from 3.0 to 38.5 fmol/ 1×10^6 cells (median, 15.4 fmol/ 1×10^6 cells) whereas ZDV-TP concentrations measured in the same population of samples were between 31.3 and 386 fmol/ 1×10^6 cells (median, 116 fmol/ 1×10^6 cells). In ZDV-treated patients, d4T-TP levels represent 0.03 to 0.37 of the ZDV-TP content. More interestingly, d4T-TP levels in these patients are clearly in the lower range of intracellular concentrations measured for patients taking d4T. This is illustrated by Fig. 3 in which intracellular d4T-TP concentrations determined in ZDV-treated patients are compared to those determined in d4T-treated patients [12]. This conclusion is particularly true for samples taken 10–14 h after dosing. Taken together, these findings seriously raise the possibility that d4T-TP could significantly participate in both the efficacy and the toxicity of ZDV.

In all of the samples examined during this study, ZDV-TP was much more concentrated than d4T-TP and, since *in vitro* experiments [13–16] have demonstrated an equivalent inhibitory effect of ZDV-TP and d4T-TP with regard to reverse transcriptase activity (K_i ranging from 8.1 to 35 nM and from 20 to 47 nM for ZDV-TP and d4T-TP, respectively), it seems very likely that the efficacy of ZDV treatment essentially lies in the intracellular production of ZDV-TP.

Similarly, one may speculate on the possibility that toxicity – in particular mitochondrial toxicity – of ZDV could result from the intracellular production of

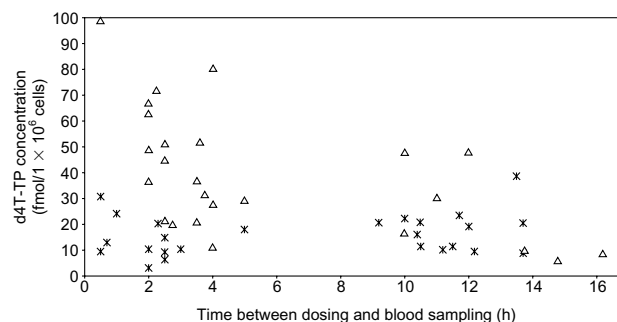


Fig. 3. Comparison of d4T-TP concentrations in patients taking ZDV (asterisks) and in patients taking d4T (triangles). d4T-TP concentrations in patients on ZDV are those in Table 1 and on d4T therapy were determined during the IMEA 012 clinical study.

d4T-TP which has been shown to be a more potent inhibitor of the human γ -polymerase responsible for the synthesis of mitochondrial DNA than ZDV-TP [17–20]. However, the fact that the spectrum of adverse events attributed to the mitochondrial toxicity of NRTI is different [17] for patients treated with ZDV from those treated with d4T (ZDV essentially induces myopathies, bone marrow toxicity and cardiopathies whereas d4T is associated mainly with the development of neuropathies and pancreatitis; both drugs induce hepatic steatosis and lactic acidosis) rather supports a major role for ZDV-TP in the mitochondrial toxicity observed in patients taking ZDV. Considering the variability of d4T-TP concentrations, one could not exclude that in certain situations, the adverse effect observed with ZDV could be attributed to both ZDV-TP and d4T-TP. This point requires re-examination.

Another characteristic features of thymidine analogues should be revisited in the light of this new finding. Several reports have shown that the set of resistance mutations observed in patients who have experienced a long period of treatment with a ZDV-containing regimen is essentially unchanged when they switch to a d4T-containing regimen, possibly explaining a diminished response to combination nucleoside therapy including d4T [21–23]. Reciprocally, ZDV resistance mutations were shown to emerge in therapy-naïve patients treated by a d4T plus didanosine combination therapy [24]. This ‘thymidine cross-resistance’ between ZDV and d4T is often evoked to explain the lack of benefit of ZDV and d4T co-administration assuming that the same set of mutations, so-called thymidine analogue mutations or TAM, confer a resistance to both NRTI. The presence of therapeutic levels of intracellular d4T-TP at trough conditions in patients on ZDV therapy could provide an alternative explanation assuming that both ZDV-TP and d4T-TP are responsible for the development of these cross-mutations. This should be an indirect demonstration that d4T-TP plays a role in the therapeutic effect of ZDV. With the current data it is not possible to decide between these two possibilities.

Clearly this work raises more questions than it answers: even if the production of significant levels of d4T-TP in patients taking ZDV therapy is well demonstrated, it is still unknown what the consequences of this new finding are in terms of efficacy and toxicity of ZDV, and the emergence of resistance mutations. However, this work shows for the first time that data obtained *in vivo* from cells isolated from patients taking antiretroviral therapy provide a better understanding of the very complex intracellular metabolism of NRTI than *in vitro* experiments. We hope that this will improve our understanding of their mechanism of action and will finally allow optimized use of NRTI at the clinical level.

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