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Decrease in Thymidylate Kinase Activity in Peripheral Blood Mononuclear Cells from HIV-Infected Individuals

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ABSTRACT. Nucleosides and nucleoside analogs are anabolised to their triphosphates by intracellular kinases. The anti-HIV analogue zidovudine (AZT) is phosphorylated by cytosolic thymidine kinase 1 (TK1), thymidylate kinase (dTMPK), and nucleoside diphosphate kinase. It is known that dTMPK is one of the rate-limiting steps in the activation of zidovudine. The activities of TK1, dTMPK, and deoxycytidine kinase (dCK) were determined in extracts of *in vitro* activated peripheral blood mononuclear cells from HIV-infected patients and healthy noninfected individuals. dTMPK activity was 10-fold lower and TK1 activity was five-fold lower in extracts from infected as compared to uninfected persons. Deoxycytidine kinase activities in the extracts from both groups were very similar. Differences in *in vitro* activation, as determined by flow cytometry, of the peripheral lymphocytes were not responsible for the decreased TK1 and dTMPK activities. A reduced level of intracellular azido-dideoxythymidinetriphosphate in activated mononuclear cells from HIV-infected patients was also observed. The low levels of TK1 and dTMPK in lymphocytes from HIV-infected patients may be related to the anergy phenomenon observed as a result of HIV infection. This effect should also be considered in the development of new anti-HIV drugs. BIOCHEM PHARMACOL **56**;3:389–395, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. antiviral agents; HIV; nucleoside phosphate kinase; nucleoside diphosphate kinase; immune tolerance

Zidovudine is still the preferred drug in the treatment of HIV infection, today used in combination with other nucleoside analogs or with nonnucleoside reverse transcriptase inhibitors and protease inhibitors [1–3]. In addition to dTMP, dTMPK,‖ dUMP, 5-Iodo-deoxyuridinemonophosphate (5-I-dUMP), [4] and the monophosphates of several thymidine analogs, e.g. azidodeoxythymidine (zidovudine, AZT) and probably didehydrodeoxythymidine (stavudine, d4T), the latter being an alternative to zidovudine in the treatment of HIV infection [3, 5]. The V_{max} for AZTMP by dTMPK is only 0.3% as compared with the V_{max} for dTMP [6]. Because cytosolic TK1 has the capacity to phosphorylate thymidine and AZT to the same extent, [6] the phosphorylation step from monophosphate to diphosphate is one important rate-limiting step in the anabolism of AZT. Furthermore, the dTMPK reaction is needed both in the salvage and the *de novo* pathway of dTTP formation and as such is crucial in the DNA precursor synthesis in all cells.

Human dTMPK is a dimer of two 23-kDa subunits whose activity increases in proliferating cells as compared to resting cells [7, 8]. However, relatively little is known about the cell-cycle regulation of dTMPK. The enzyme is very unstable *in vitro* and the presence of substrates, reducing agents and detergents stabilise the activity [9, 10].

In our earlier studies of TK levels in PBMC, we found a decreased level of TK in cells from HIV-infected individuals [11]. This evidently affects all PBMC although only a fraction of the cells are infected by HIV. This result indicated that decreased TK activity could be part of a host cell drug resistance phenomenon. Avramis *et al.* described a T-cell line (Jurkat E6-1) grown in continuously increasing concentrations of AZT from 1 to 100 μM as showing less growth inhibition by the drug after 10 weeks in culture. The phenomenon was apparently due to decreasing intracellular levels of TK with subsequently decreased AZTTP formation. It was demonstrated that patients treated with zidovudine had a very large variation in the intracellular levels of phosphorylated zidovudine, although there was a direct relationship between the serum drug level and the intracellular levels of phosphorylated zidovudine [12]. It was shown that treated patients exhibit a progressively decreas-

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‖ *Abbreviations:* AZT, 3′-azido-2′,3′-dideoxythymidine (zidovudine); AZTTP, azido-dideoxythymidinetriphosphate; dCK, deoxycytidine kinase; ddC, 2′,3′-dideoxycytidine; dThd, 2′-deoxythymidine; dTMPK, thymidylate kinase (deoxythymidinemonophosphase kinase); NDPK, nucleoside diphosphate kinase; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; and TK1, thymidine kinase 1.

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ing intracellular concentration of phosphorylated AZT over time [13].

The aim of the present study was to determine the levels of key enzymes involved in the phosphorylation of AZT, using both the natural nucleoside as well as the analogue as substrate. We found a significant difference in the levels of TK1 and dTMPK in the extracts of PBMC from HIV-infected patients and noninfected individuals, but no difference was observed in the level of dCK.

MATERIALS AND METHODS

Chemicals

AZT, dThd, and 2-chlorodeoxyadenosine were obtained from Sigma. Azido-[methyl-^{3}H]-dideoxythymidine, [methyl-^{3}H]-deoxythymidine, and [methyl-^{3}H]-2-chlorodeoxyadenosine were purchased from Moravek Biochemicals Inc. PHA was obtained from Welcome Diagnostics. RPMI was purchased from Flow and PEI-Cellulose F paper was purchased from Merck.

Subjects

PBMC were isolated from healthy HIV-seronegative (HIV^-) and HIV-seropositive (HIV^+) individuals who had earlier been included in studies on the intracellular phosphorylation of AZT and TK levels [11, 14]. dTMPK was assayed in extracts from 38 different people, 11 healthy HIV^- and 27 HIV^+ individuals in different stages of the infection, from asymptomatic to patients with AIDS. The AZT-treated group was subdivided into two groups, one treated for less than 17 months and the other treated for 17 months or more. We also tested 5 HIV^+ individuals who showed intolerance to AZT in the form of transfusion-dependent anemia after less than 6 months of zidovudine therapy at a dose of no more than 600 mg/day.

dCK was assayed in 95 PBMC extracts from 65 individuals of whom 20 were HIV-seronegative. The T-lymphocyte cell subsets were determined by FACScan analysis.

Isolation of PBMC and Cell Culture

PBMC were isolated from vein blood and separated on Ficoll-paque gradients. The isolated cells were stimulated with PHA and incubated at 37° for 72 hr in a humidified 5% CO_2 atmosphere in growth medium. The cells were harvested and the enzymes extracted with Triton X-100 as described [11]. The distribution of cells in different cell cycle phases was determined by flow cytometry analysis of ethanol-fixed cells (performed by S. Skog and B. Tribukait, Department of Radiobiology, Karolinska Institute, Stockholm) [15].

Protein Content

The amount of protein in each extract was determined by the BCA protein assay reagent from Pierce.

Preparation of the Deoxythymidine Monophosphate Substrate

Human recombinant TK1 [16] was added to a cocktail with a final composition as follows: 1 mM ATP, 1 mM $MgCl_2$, 50 mM Tris (pH 7.6), 0.5 mg/mL of BSA, 1 mM of dithiothreitol, 50 mM KCl, 45 μM dThd, and 5 μM ^{3}[H]-dThd. The reaction was performed at 37° for 4 hr and was stopped by boiling for 2 min. The mixture was centrifuged and denatured proteins were removed, and the nucleotides in the supernatant were then separated by DEAE chromatography as follows. The supernatant was diluted with NH_4HCO_3 to a final concentration of 0.5 mM and applied to a DEAE sepharose column equilibrated in the same buffer. The nucleotides were subsequently eluted with an increasing concentration of NH_4HCO_3 from 10–500 mM. The deoxythymidine monophosphate eluted at a concentration of 50–75 mM NH_4HCO_3.

^{3}[H]-azidothymidine was prepared according to the same protocol except that ^{3}[H]-AZT was substituted for ^{3}[H]-dThd.

dTMPK Assay

dTMPK activity was determined with thymidine monophosphate as substrate as follows: enzyme extracts from stimulated cells (25–100 μg of protein) were diluted in H_2O to a volume of 100 μL and the reaction was immediately started by adding a reaction cocktail (25 μL) to react a final concentration of 5 mM ATP, 50 mM Tris-HCl pH 7.6, 0.1 mg/mL of BSA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 50 μM [^{3}H]dTMP (approximately 2000 cpm/pmol). The reactions were performed at 37° and aliquots of 25 μL were withdrawn and applied to Watman DE-81 filter discs at 0, 20, 40, and 60 min respectively. The filter discs were washed twice in 4 M formic acid plus 1 mM of ammonium formate and once in 96% ethanol, and eluted with 0.1 M HCl plus 0.2 M KCl as described [17]. Scintillation fluid was added and the radioactivity determined.

The product of the reaction was also analysed with TLC. Samples were incubated as above for 60 min and boiled for 2.5 min to stop the reaction. The samples were centrifuged and cell debris was removed. A portion of the supernatant was spotted onto TLC cellulose paper with dThd, dTMP, dTDP and ATP as markers. TLC was performed with 2% boric acid/2 M LiCl (65:35, v/v) [18]. The separated dTMP, dTDP, dTTP and dThd spots were eluted with 0.1 M HCl plus 0.2 M KCl, and the radioactivity of these spots was determined. As expected, there was very close correlation when the dTMPK assay was measured with TLC or by the filter disc method (not shown).

To rule out any influence of inhibiting factors in samples with low activity, three samples with high activity were individually mixed with three samples with low activity and assayed as described.

In six samples with high dTMPK activity, enzyme activity was also determined with AZTMP as substrate. Extracts

(86-450 μg of protein) were incubated under the reaction conditions described above, and the concentration of azidodeoxythymidine monophosphate was 50 μM. The reaction was in this case performed for 30, 60, 120, and 180 min.

AZT Metabolism in Isolated BMC

Peripheral blood mononuclear cells from 17 seronegative individuals and three HIV-infected patients were isolated and incubated with azidothymidine. In short, isolated PBMC, stimulated with PHA, were incubated with 1.6 μM AZT for 4 hr. The nucleotides were subsequently extracted with methanol and analysed by ion exchange HPLC as described earlier [14].

dCK Kinase Assay

dCK activity was determined with ^{3}[H]-2-chlorodeoxyadenosine (CldA) and enzyme extract (50 μg of protein) as described [19].

Statistical Analysis

The distribution of the dTMPK activity values was found to be skewed to the left. In order to perform a valid statistical analysis, these values were subsequently substituted with their natural logarithms. dTMPK activity, dCK activity, and AZT phosphorylation were expressed as median ± 25 percentiles. Data from two different groups were compared using Student's *t*-test (two-tailed). Data from more than two groups were compared with ANOVA with a 95% confidence interval. A regression analysis was performed to obtain the correlation between different enzyme activity values and the cell-cycle phase composition.

RESULTS

Subjects and Cell-Cycle Distribution of PBMC Samples

Patients attending the infectious clinic at Roslagstulls Hospital and Huddinge Hospital, Karolinska Institute, Stockholm, Sweden were included after informed consent. The patients represented an unselected group of HIV-infected people attending an infectious clinic in Sweden in 1991 and 1992. The CD 4 cells of the patients from whom the peripheral blood samples were collected varied between 5 and 400 × 10^6/L. Nine had no previous AZT treatment, four were treated for between 1 and 16 months, and 8 were treated for more than 17 months. CD4 and CD8 cell counts, AZT treatment, and dTMPK activities of the HIV-infected patients are listed in Table 1. The cell-cycle composition was determined and the mean values of all samples at time of harvest were: 58% ± 13 in G1, 32% ± 10 in S, and 9% ± 4 in G2. The cells described as G1 were composed of G0 and G1 cells.

Stability of dTMPK and dCK

In order to establish the experimental variation in the dTMPK assay, frozen crude extracts from PHA-stimulated PBMC's were diluted four-fold with H_2O and subsequently analysed. The dilution did not lead to any inhibition or stimulation of enzyme activity. Freezing and thawing lowered the dTMPK activity approximately 8%, and the activity was decreased by 90% after 72 hr when stored at 4°. At room temperature 22°, the activity was decreased by 90% after 18 hr.

The stability of the dCK activity was established after repeated freezing, which lowered the activity by approximately 5% for each freezing cycle. The activity decreased 10% after 24 hr when stored at 4°. However, the specific activity of dCK reported here was lower than observed earlier [19]. We used 2% Triton X-100 in the extraction buffer instead of 0.5% NP 40, which was used earlier. The presence of detergents affects the substrate specificity of dCK [20], and the high level of Triton X-100 used in this case may have inhibited the enzyme.

dTMPK Activity in Extracts from Activated PBMC

The dTMPK reaction was linear for 60 min at 37° except with extracts with a low activity, where there was a delay before the reaction reached a linear range (after approximately 20 min of preincubation). The dTMPK activities in the growth medium were barely detectable (5 pmol/mg × min), which showed that dTMPK is an intracellular protein.

dTMPK activity in PBMC extracts showed a very large variation. The median activity was 300 [127–464] pmol/mg × min. When it was related to the proportion of S-phase cells, it was 9.1 (4.3–18) pmol/mg × min × % S-phase in extracts from noninfected individuals. In extracts from HIV-infected individuals, it was 27 pmol/mg × min [11–73] and 0.87 (0.46–1.9) pmol/mg × min × % S-phase (Tables 1–3). This difference is statistically significant (Student's *t*-test $p < 0.001$). Patients treated with AZT for a prolonged period did not show lower activity [0.89 (0.29–1.1) pmol/mg × min × % S-phase] as compared to untreated patients [0.87 (0.72–2.8) pmol/mg × min × % S-phase]. Patients with intolerance to AZT showed a similar dTMPK activity [0.59 (0.42–1.8) pmol/mg × min × % S-phase] to that of other patients (Table 3).

In samples with high dTMPK activity, there was only minor accumulation of dTDP during the assay as determined by TLC analysis. Conversely, dTTP increased by a factor of 27 while dTDP only increased eight-fold. There was no apparent degradation of phosphorylated deoxythymidine to deoxythymidine during the assay as judged from TLC analysis.

Four extracts from nonstimulated PBMC of HIV-seronegative subjects were tested in the same assay; in contrast to TK [21], a low but significant dTMPK activity was

TABLE 1. CD 4 and CD 8 cell count and duration of AZT treatment and dTMPK activities in HIV-seropositive patients

Patient no.	CD4 cells 10^6/L	CD8 cells 10^6/L	AZT treatment months	dTMPK pmol dTDP formed/min/mg	dTMPK pmol dTDP formed/min/mg × %S
1	5	100	intolerant	7.6	0.34
2	6	300	23	47	1.47
3	15	400	17	36	1.11
4	20	600	11	20	4.92
5	20	400	19	53	0.83
6	30	800	0	22	0.73
7	40	1100	22	58	0.96
8	40	400	intolerant	24	0.46
9	45	800	21	9.7	0.16
10	80	700	15	145	2.07
11	110	700	8.5	8.6	0.14
12	130	1800	intolerant	53	0.72
13	150	700	intolerant	16	0.44
14	160	700	17.5	194	1.88
15	160	700	2	73	1.06
16	180	300	0	161	1.97
17	200	400	0.5	189	2.16
18	240	1500	0	23	0.34
19	240	400	0	84	0.87
20	250	600	0	68	0.71
21	250	800	0	156	1.80
22	270	1600	0	159	3.34
23	320	1600	30	27	0.47
24	350	400	0	67	0.73
25	360	600	intolerant	116	1.78
26	400	700	0	500	5.60
27	n.d.	n.d.	36	17	0.23

n.d. = not determined.

detected (approximately 10% of that observed with stimulated cells) (data not shown). Whether this was due to the same or a different form of dTMPK is not known.

There was no decrease in the activity of the samples with high activity when a sample with low activity was added to it. The activities in the mixed extracts demonstrated that there were no inhibitors of dTMPK in extracts from HIV-infected patients.

dTMPK Activity and AZT Phosphorylation

dTMPK has a low capacity to phosphorylate AZTMP. Therefore, it was not possible to perform a complete set of assays with all the samples using AZTMP as substrate. However, when extracts with high dTMPK activity were used and the assay was performed for a longer time period (180 min), it was possible to detect AZTMP phosphorylation. The reaction was linear for 180 minutes at 37°. Six extracts were tested in this way, and there was a very good correlation between the observed dTMP and AZTMP phosphorylation (Fig. 1). The activity in the extracts varied from 80 to 825 pmol/mg × min. The activity of dTMPK was approximately 0.5% when AZTMP was used instead of dTMP, which is in accordance with earlier results [6].

TABLE 2. dTMPK activities in HIV-seronegative individuals

HIV-seronegative individual	dTMPK pmol dTDP formed/min/mg	dTMPK pmol dTDP formed/min/mg × %S
1	247	5.9
2	114	n.d.
3	264	n.d.
4	181	n.d.
5	86	n.d.
6	628	21
7	541	18
8	65	2.2
9	56	1.1
10	429	15
11	355	11
12	226	7.2
13	407	17
14	134	4.2
15	124	4.9
16	79	2.0
17	476	19
18	148	4.7
19	230	6.4
20	569	18
21	361	11
22	408	16
23	723	23
24	103	2.3

n.d. = not determined.

TABLE 3. Enzyme activity in extracts of PHA-activated PBMC from HIV-seropositive patients and-seronegative individuals

Extracts	N	dTMPK pmol dTDP formed/min/mg	dTMPK pmol dTDP formed/min/mg × %S	dCK pmol CdAMP formed/min/mg	dCK pmol CdAMP formed/min/mg × %S
HIV neg	20	300 (127–464)	9.1 (4.3–18)	39 (17–55)	0.94 (0.42–1.8)
HIV pos	27	27 (11–73)	0.87 (0.46–1.9)	29 (13–35)	0.74 (0.40–1.2)
HIV pos no AZT*	9	42 (23–87)	0.87 (0.72–2.8)		
HIV pos AZT > 17 mon†	8	21 (9.8–28)	0.89 (0.29–1.1)		
AZT intol‡	5	19 (6.9–64)	0.59 (0.42–1.8)		

The activity is expressed as pmol product formed per mg of protein and min, and the values are median ± 25th percentile in parenthesis.
*HIV-positive patients with no prior AZT medication.
†Patients treated with AZT for more than 17 months.
‡Patients with AZT intolerance in the form of transfusion-dependent anemia.

AZT Metabolism in Isolated PBMC

In order to determine if the reduced levels of TK1 and dTMPK were correlated to a decreased capacity of PBMC to anabolise AZT, a set of experiments was performed in which the intracellular concentration of AZT nucleotides was measured using HPLC analysis [14]. The extent of phosphorylation of AZT to the intracellular AZTMP, AZTDP, and AZTTP was significantly lower in PBMC from HIV-infected persons compared to cells from healthy individuals. In cells from healthy individuals incubated with 1.6 μM AZT, we found 0.27 (0.17–0.38) pmol of AZTTP/10^6 cells compared to 0.007 (0.004–0.01) pmol/10^6 cells in cells from HIV-infected individuals (statistical significance: $P = 0.029$). In addition, the level of AZTTP compared to the totally phosphorylated AZT nucleotides was decreased in cells from HIV-infected individuals (Table 4).

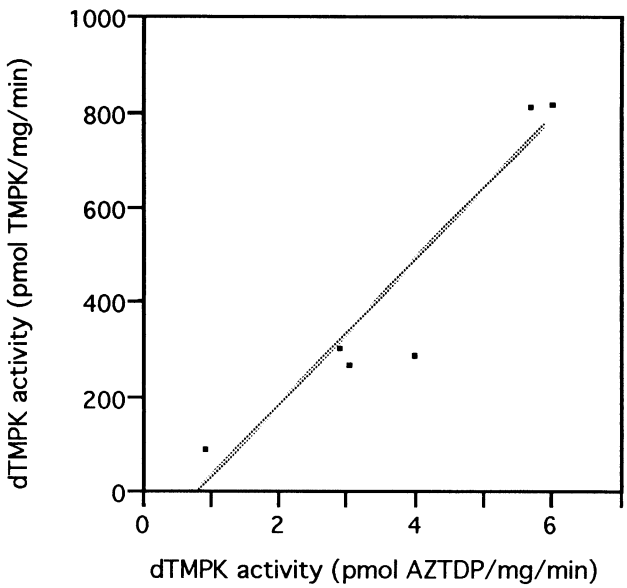

FIG. 1. Correlation of the activity of dTMPK determined with dTMP or AZTMP as substrates. $r^2 = 0.88$ and $P = 0.0053$.

dCK Activity in Extracts from Activated PBMC Samples

dCK activities determined in the PBMC extracts also showed a very large variation. The median value was 27 pmol/mg/min and when related to S-phase cells was 0.78 pmol/mg × min × % S-phase. There was no difference between the levels in extracts from HIV^- and HIV^+ individuals, i.e., 0.94 (0.42–1.8) and 0.74 (0.40–1.2) pmol/mg × min × % S-phase, respectively. The dCK activity was to some extent related to the cell-cycle composition of the PBMC, which is in agreement with earlier studies [19, 22]. Patients not treated with AZT had the same levels of dCK as patients treated >17 months as well as patients who showed AZT intolerance.

DISCUSSION

dTMPK was higher in extracts from stimulated PBMC from healthy individuals as compared to extracts from cells isolated from HIV-infected patients. Both TK1 and dTMPK were low in samples from patients with a CD_4 count below 400 [11]. In a few samples from patients with a higher CD_4 count, there was a tendency to find higher TK1 activity, close to that of noninfected persons. The dTMPK activity was not correlated to the CD4 cell counts of the patients. The lower activity noted in PBMC from HIV-infected individuals is most likely not due to the presence of inhibitors, because in the mixing experiment no indications for the presence of inhibiting substances were found. *In vitro* experiments with PBMC infected with HIV-1 demonstrated a 50% decrease in TK1 activity in the extracts [23]. Recent experiments confirm this result and a decrease in dTMPK activity was also observed. Further work with *in vitro* HIV-infected PBMC is now in progress.

We speculate that the down regulation of enzymes regulated by the cell cycle during DNA synthesis may be related to the anergy phenomenon associated with HIV infection [24]. The anergy observed in HIV infection is usually noted when the CD_4 count decreases to a value approximate to or below 400 × 10^6/L, which is in agree-

TABLE 4. Rate of phosphorylation of AZT to AZTTP in PHA-stimulated PBMC from HIV-infected patients compared to healthy individuals

	Number	AZT in medium	AZTMP pmol/10^6 cells	AZTDP pmol/10^6 cells	AZTTP pmol/10^6 cells	% ZTTP of phosphorylated AZT
HIV neg median ± 25th percentile	17	1.6 μM	64 (43–78)†	0.36 ± 0.43	0.27 (0.17–0.38)*	0.4 (0.25–0.5)‡
HIV pos median and range	3	1.6 μM	5.2 (0.6–17.7)†	0.11 ± 0.13	0.007 (0.004–0.01)*	0.05 (0.0–0.1)‡

The differences are statistically different.
*$P = 0.029$.
†$P = 0.014$.
‡$P = 0.006$.

ment with the decreased TK and dTMPK activities found here. However, there was not a general decrease in DNA salvage enzyme activities in these cells, since the dCK levels were similar in all extracts. Thus, selectively decreased TK 1 and dTMPK levels might be biochemical markers for the hyporesponsiveness of the PBMC from HIV-infected patients.

Although only a minority of all the PBMC are HIV-1-infected [24] the dTMPK activity in the extracts was only 10% as compared to extracts from uninfected individuals. We therefore conclude that this phenomenon is not restricted to HIV-infected cells, but is a generalised effect occurring in a large fraction of the PBMC from HIV-infected persons. The proliferation test with mononuclear cells from HIV-infected individuals showed a 3- to 5-fold decrease in thymidine incorporation as compared to cells from noninfected people upon stimulation with PHA, tuberculin, morbilli, and tetanus.* This result is a consequence of the above mentioned anergy phenomenon and is in agreement with the hyporesponsiveness of the CD 8 cells from HIV-infected patients described recently [25].

The finding that dTMPK activity is decreased in cells from HIV-infected individuals is most likely more important than the decrease noted in TK1 activity. However, the results in Table 4 indicate that the NDPK reaction is also limiting in cells from HIV-infected individuals. Recent results show that the NDPK reaction is 0.5% with AZTDP as substrate compared to TDP, and thus both dTMPK and NDPK may be considered as rate-limiting enzymes in AZT anabolism. [26, 27]. Activated PBMC from HIV-infected patients incubated with AZT showed a significantly decreased amount of AZT phosphorylated to AZTTP as compared to healthy individuals ($P = 0.029$). The anabolism of azidothymidine was also decreased in HIV-infected H9 cells [6]. Taken together, these results indicate that decreased TK1 and dTMPK activities may also reduce the anabolism of AZT in intact cells. Although there is a decreased activation of AZT in HIV-infected cells, the concentration of intracellular AZTTP achieved leads to inhibition of HIV RT and blocked virus production because AZTTP shows very high affinity for HIV RT [28].

* Jacobbson B, Britton S, Immunological effects during DNCB application of HIV-infected patients, manuscript in preparation.

The mechanisms by which HIV infection down-regulates both TK and dTMPK is not known. TK1 regulation occurs both at the transcriptional and/or the translational level, and there is at present no published information regarding the transcriptional control of the dTMPK gene. During zidovudine treatment, it has been shown that TK was down-regulated by hypermethylation of the TK promotor [29, 30]. Such a mechanism may also be involved in dTMPK gene regulation. Many test systems for new antiviral drugs are based on *in vitro* culture of cell lines or cells from healthy blood donors. In view of the results presented here, these results must be used with caution since the metabolism of nucleoside analogs differs in cells from healthy individuals compared to HIV-infected individuals.

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References

1. Fischl MA, Richman DD, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Schooley RT, Jackson GG, Durack DT, Kingand D and the AZT collaborative group, The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. *N Engl J Med* **317:** 185–191, 1987.
2. Mitsuya H, Weinhold KJ, Furman PA, St Clair M, Lehrman SN, Gallo RC, Bolognesi D, Barry DW and Broder S, 3′-Azido-3′-deoxythymidine (BW A509U), an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc Natl Acad Sci USA* **82:** 96–100, 1985.
3. BHIV Guidelines Co-ordinating Committee, British HIV Association guidelines for antiretroviral treatment of HIV seropositive individuals. *Lancet* **349:** 1086–1042, 1997.
4. Jong AY and Campbell JL, Characterization of Saccharomyces cerevisiae thymidylate kinase, the CDC8 gene product. General properties, kinetic analysis, and subcellular localization. *J Biol Chem* **259:** 14394–14398, 1984.
5. Lin TS, Schinazi RF and Prusoff WH, Potent and selective *in vitro* activity of 3′-deoxythymidin-2′-ene (3′-deoxy-2′,3′-

didehydrothymidine) against human immunodeficiency virus. *Biochem Pharmacol* **36:** 2713–2718, 1987.
6. Furman PA, Fyfe JA, St Clair MH, Weinhold K, Rideout JL, Freeman, GA, Lehrman SN, Bolognesi DP, Broder S, Mitsuya H and Barry DW, Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc Natl Acad Sci USA* **83:** 8333–8337, 1986.
7. Koonin EV and Senkevich TG, Evolution of thymidine and thymidylate kinases: the possibility of independent capture of TK genes by different groups of viruses. *Virus Genes* **6:** 187–196, 1992.
8. Su JY and Sclafani RA, Molecular cloning and expression of the human deoxythymidilate kinase gene in yeast. *Nucleic Acids Res* **19:** 823–827, 1991.
9. Lee LS and Cheng Y, Human thymidylate kinase. Purification, characterization, and kinetic behavior of the thymidylate kinase derived from chronic myelocytic leukemia. *J Biol Chem* **252:** 5686–5691, 1977.
10. Bojarski TB and Hiatt HH, Stabilization of thymidine kinase activity by thymidylate and by thymidine. *Nature* **188:** 1112–1114, 1960.
11. Jacobsson B, Britton S, He Q, Karlsson A, and Eriksson S, Decreased thymidine kinase levels in peripheral blood cells from HIV-seropositive individuals: Implications for zidovudine metabolism. *AIDS Res Hum Retroviruses* **11:** 805–811, 1995.
12. Stretcher BN, Pesce AJ, Hurtubise PE and Frame PT, Pharmacokinetics of zidovudine phosphorylation in patients infected with the human immunodeficiency virus. *Ther Drug Monit* **14:** 281–285, 1992.
13. Stretcher BN, Pesce AJ, Frame PT and Stein DS, Pharmacokinetics of zidovudine phosphorylation in peripheral blood mononuclear cells from patients infected with human immunodeficiency virus. *Antimicrob Agents Chemother* **38:** 1541–1547, 1994.
14. Tornevik Y, Jacobsson B, Britton S and Eriksson S, Intracellular metabolism of 3′-azidothymidine in isolated human peripheral blood mononuclear cells. *Aids Res Hum Retroviruses* **7:** 751–759, 1991.
15. Tribukait B, Moberger B and Zetterberg A, Methodological aspects on rapid-flow cytofluorometry for DNA-analysis of human urinary bladder cells. In: *First International Symposium on Pulse Cytophometry* (Ed. Wessel HH), p 50 [Abstract], Ghent, Belgium, 1975.
16. He Q, Skog S, Wang N, Eriksson S and Tribukait B, Characterization of a peptide antibody against a C-terminal part of human and mouse cytosolic thymidine kinase, which is a marker for cell proliferation. *Eur J Cell Biol* **70:** 117–124, 1995.
17. Chen MS, Chang PK and Prusoff WH, Photochemical studies and ultraviolet sensitization of *Escherichia coli* thymidylate kinase by various halogenated substrate analogs. *J Biol Chem* **251:** 6555–6561, 1976.
18. Tamiya N, Yusa T, Yamaguchi Y, Tsukifuji R, Koroiwa N, Moriyama Y and Fujimura S, Co-purification of thymidylate kinase and cytosolic thymidine kinase from human term placenta by affinity chromatography. *Biochim Biophys Acta* **995:** 28–35, 1989.
19. Arner ES, Spasokoukotskaja T and Eriksson S, Selective assays for thymidine kinase 1 and 2 and deoxycytidine kinase and their activities in extracts from human cells and tissues. *Biochem Biophys Res Commun* **188:** 712–718, 1992.
20. Kierdaszuk B and Eriksson S, Selective inactivation of the deoxyadenosine phosphorylating activity of pure human deoxycytidine kinase: stabilization of different forms of the enzyme by substrates and biologic detergents. *Biochemistry* **29:** 4109–4114, 1990.
21. Bello LJ, Regulation of thymidine kinase synthesis in human cells. *Exp Cell Res* **89:** 263–274, 1974.
22. Starnes MC and Cheng YC, Cellular metabolism of 2′,3′-dideoxycytidine, a compound active against human immunodeficiency virus *in vitro*. *J Biol Chem* **262:** 988–991, 1987.
23. Arner ES, Valentin A, and Eriksson S, Thymidine and 3′-azido-3′-deoxythymidine metabolism in human peripheral blood lymphocytes and monocyte-derived macrophages. *J Biol Chem* **267:**10968–10975, 1992.
24. Yerly S, Chamot E, Hirschel B and Perrin LH, Quantitation of human immunodeficiency virus provirus and circulating virus: Relationship with immunologic parameters. *J Infect Dis* **166:** 269–276, 1992.
25. Vingerhoets J, Kestens L, Penne G, De Vuyst H, Vandenbruaene M, Pelgrom Y, Bosmans E, De Boer M, Kasran A, Azuma M, Colebunders R, Ceuppens JL and Vanham G, CD 8^+ T cells and not CD 4^+ T cells are hyporesponsive to CD 28- and CD 4-DL-mediated activation in HIV-infected subjects. *Clin Exp Immunol* **107:** 440–447, 1997.
26. Lavie A, Schlichting I, Vetter IR, Konrad M, Reinstein J and Goody RS, The bottleneck in AZT activation. *Nature Med* **3:** 922–924, 1997.
27. Bourdais J, Biondi R, Sarfati S, Guerreiro C, Lasco I, Janin J and Veron M, Cellular phophorylation of anti-HIV nucleosides. *J Biol Chem* **271:** 7887–7890, 1996.
28. Lacey SF, Reardon JE, Furfine ES, Kunkel TA, Bebenek K, Eckert KA, Kemp SD and Larder BA, Biochemical studies on the reversed transcriptase and RNase H activities from human immunodeficiency virus strains resistant to 3′-azido-3′-deoxythymidine. *J Biol Chem* **267:** 15789–15794, 1992.
29. Wu S, Liu X, Solorzano MM and Kwock R, Development of zidovudine (AZT) resistance in Jurkat T cells is associated with decreased expression of the thymidine kinase (TK) gene and hypermethylation of the 5′ end of human TK gene. *J Acquired Immune Defic Syndr* **8:** 1–9, 1995.
30. Avramis VI, Kwock R, Solorzano MM and Gomperts E, Evidence of *in vitro* development of drug resistance to azidothymidine in T-lymphocytic leukemia cell lines (Jurkat E6–1/AZT-100) and in pediatric patients with HIV-1 infection. *J Acquired Immune Defic Syndr* **6:** 1287–1296, 1993.