Thymidine kinase and deoxycytidine kinase activity in mononuclear cells from antiretroviral-naive HIV-infected patients

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Objective: To evaluate whether an inter-individual variability in the activity of thymidine kinase (TK) and deoxycytidine kinase (dCK), which are involved in the first step of phosphorylation of some nucleoside analogues, exists in antiretroviral-naive, HIV-seropositive patients.

Design: Forty-five randomly selected antiretroviral-naive HIV-infected patients were recruited, together with 26 healthy volunteers with no concurrent infection and under no pharmacological treatment.

Methods: Peripheral blood mononuclear cells (PBMC) were isolated from venous blood and their TK and dCK activities evaluated. CD4 T cells and HIV-RNA were measured in HIV-infected patients, too.

Results: There was a broad range of variability in TK activity in HIV-infected individuals. Furthermore, the activity in PBMC was significantly higher in HIV-infected individuals than in healthy volunteers. dCK activity in seropositive patients was significantly lower than in healthy volunteers. A marked inter-individual variability in dCK levels was observed in the HIV-infected group. No correlations were found between TK or dCK activities and plasma viral load, CD4 cell count, sex or age of patients.

Conclusions: A marked range of inter-individual variability of TK and dCK activities in PBMC exists in HIV-infected individuals but not in healthy volunteers, indicating that the activity of enzymes with key roles in drug activation could vary greatly from one patient to another. Furthermore, TK expression is greater in HIV-infected individuals than in healthy volunteers. Better understanding of the viral or cellular factors that contribute to this variability, as well as their effect on responses to antiretroviral treatment, may aid optimization of the management of HIV-infected patients.

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Introduction

Heterogeneity in the response to antiretroviral treatment for HIV infection is a phenomenon well described in the literature. The variability in response to therapy has been attributed to virological, immunological and pharmacological factors [1–3].

From a pharmacological point of view, it is well known that the antiviral activity of treatment drugs depends strictly on their concentration at the site of action [4]; differences in individual ability to metabolize these drugs may influence systemic and intracellular drug levels, and may possibly result in variability in response to the antiretroviral therapy.

Several authors have evaluated the possibility of employing therapeutic drug monitoring (TDM) as a tool to individualize and optimize antiretroviral therapy in HIV-infected patients [5–7]. In general, such a strategy can be applied to treatments that exhibit a close correlation between concentrations in plasma and at the site of action. In HIV therapy, protease inhibitors appear to be the best candidates for TDM and most of the studies in which the concentration–effect relationship has been described were performed using these drugs [5–8]. In contrast, TDM has limited value for nucleoside reverse transcriptase inhibitors (NRTI). Indeed, the active form of NRTI is not the parent drug but the anabolite triphosphate that is formed intracellularly by a phosphorylation process mediated by cellular kinases; thus, plasma levels of the parent drug cannot be considered a good indicator of intracellular triphosphate concentrations. Different approaches have been made to addressing the issue of the intracellular pharmacology of NRTI [9–12]. For example, in a study of HIV-infected individuals it was reported that, during therapy, the intracellular concentrations of zidovudine triphosphate and lamivudine triphosphate were related to the percentage change in CD4 cells and to the rate of decline in HIV RNA in plasma [11]. Data from an *in vitro* study demonstrated a correlation between the pharmacological activation of nucleoside analogues and the suppression of HIV replication [12]. It has also been observed that the response to therapy is better when drugs are administered by concentration-controlled methods and that there is considerable inter-individual variability in the intracellular concentration of NRTI [9,10]. These data support the concept that between-patient pharmacological variability may affect the anti-HIV response. Such variability could be due not only to the systemic concentration of the parent drug but also to the activity of the enzyme involved in the dideoxynucleoside phosphorylation on which the activation state of the target cells depends. For example, didanosine, lamivudine and zalcitabine have been classified as cell cycle activation-independent dideoxynucleosides since they produce higher ddNTP/dNTP ratios and exert more potent antiretroviral activity in resting cells [13–16], whereas zidovudine and stavudine are preferentially phosphorylated in activated cells [13–16]. More specifically, zidovudine and, presumably, stavudine are converted to the 5′-monophosphate level by cytosolic thymidine kinase (TK), a cell cycle-regulated enzyme [17]. The activity of this cellular enzyme fluctuates with DNA synthesis, being high in dividing cells and low in quiescent cells. In comparison, the deoxycytidine (dCyd) analogues, whose phosphorylation is catalysed by cytosolic dCyd kinase (dCK), show an antiviral activity that is independent of the cellular cycle, the activity being expressed both in resting and stimulated lymphocytes [13–16].

The aim of this study was to evaluate the extent of the variability in the cellular enzymes involved in the first step of dideoxynucleoside phosphorylation in HIV-infected, antiretroviral-naive patients. We chose to investigate TK and dCK because these enzymes catalyse the phosphorylation of the main NRTI used in HIV treatment, such as zidovudine and lamivudine, and because TK is an S-phase-dependent enzyme while dCK activity is independent of cell activation.

Methods

Chemicals

[Methyl-^{3}H]-zidovudine ([^{3}H]-zidovudine, 15.1 Ci/mmol) was from Moravek Biochemicals (Brea, California, USA). [Methyl-^{3}H]-thymidine ([^{3}H]-Thd, 25 Ci/mmol) and [^{3}H]-2'-dCyt ([^{3}H]-dCyd, 22 Ci/mmol) were from Amersham (Milan, Italy).

Participants

Samples of peripheral blood mononuclear cells (PBMC) were isolated from 45 randomly selected, antiretroviral-naive, HIV-infected individuals [18 women and 27 men, median age 35 years (range, 26–56 years)] and from 26 volunteers (12 men and 14 women, median age 39 years (range, 23–52 years)]. The healthy donors recruited for this study showed no concurrent infection and were undergoing no pharmacological treatment.

The median CD4 cell count in the HIV-seropositive group was 423×10^6 cells/l (range, $40–1030 \times 10^6$ cells/l). The HIV RNA median was 32 670 copies/ml (range, 820–1.5×10^6 copies/ml). Of the 45 patients, 41 were Caucasian, three were Africans and one was South American. Their characteristics are summarized in Table 1.

All patients gave their written informed consent before participation in the study.

Isolation of PBMC

PBMC were isolated from approximately 10 ml of venous blood and separated on Lympholyte-H (Cedarlane

Table 1. Characteristics of the individuals included in the study.

	HIV-infected patients	Healthy individuals
Total numbers	45	26
Gender		
Male	28	12
Female	17	14
Median age (years)	35 (range, 26–56)	39 (range, 23–52)
CD4 T lymphocytes (1×10^6cells/l)	432 (range, 40–1030)	N.A.
Plasma HIV-RNA (copies/ml)	32,670 (range, 820–1.5×10^6)	N.A.

N.A., Not applicable.

Laboratories Ltd, Hornby, Ontario, Canada) gradients. The isolated cells were pelleted by centrifugation, washed twice with ice-cold, phosphate-buffered saline and stored at −80°C until required.

Preparation of cellular extracts for enzymatic activity

The PBMC pellets ($8-10 \times 10^6$ cells) were resuspended in 5 vols 25 mM Tris–HCl (pH 8), 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (DTT), sonicated three times at 50 W for 5 s and centrifuged at 10 000 rpm in an Eppendorf centrifuge at 4°C for 20 min. The supernatants from each sample were divided into several tubes and frozen at −80°C until required.

Protein concentrations in the extracts were measured using the Bio-Rad (Richmond, California, USA) protein assay, with bovine serum albumin (Sigma, Milan, Italy) as the standard.

TK assay

In the TK assay of the supernatant, the diethylaminoethanol (DEAE) cellulose disc method was used [18]. Briefly, varying amounts of extracts were incubated at 37°C for 20 min in 25 μl of a mixture of 30 mM HEPES-K+ (pH 7.5), 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM DTT, and 1.6 μM [^{3}H]-Thd. The reaction was terminated by spotting 20 μl of the incubation mixture on to a 25-mm DEAE disc (DE-81 paper disc: Whatman, Clifton, New Jersey, USA). The disc was washed three times in an excess of 1 mM ammonium formate pH 3.6, to remove any unconverted nucleoside, and then, finally, in ethanol. The level of radioactive thymidine monophosphate ([^{3}H]-TMP) was estimated by scintillation counting in 4 ml of Ultima Gold scintillating fluid (Packard BioSciences, Groningen, the Netherlands). Under these conditions, one unit of TK was defined as the amount of enzyme that converted 1 nmol of [^{3}H]-Thd into [^{3}H]-TMP/h. The assay was repeated using radiolabelled zidovudine (2.6 μM) as a substrate.

2′-dCK assay

The dCK activity present in the PBMC extracts was assayed using a radiochemical method that measures the formation of [^{3}H]-dCyd monophosphate ([^{3}H]-dCMP) from [^{3}H]-dCyd. The cellular extracts were incubated at 37 °C in 25 μ of a mixture containing 30 mM HEPES-K+ pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mM DTT and 2.4 μM [^{3}H]-dCyd (1.8 μM). After a 20 min incubation, the reaction was terminated by spotting 20 μl of the incubation mixture on to a 25-mm DEAE disc (DE-81 paper disc). The discs were washed three times in an excess of 1 mM ammonium formate pH 3.6, to remove any unconverted nucleoside, and then finally in ethanol. After drying the discs, radioactive dCMP was estimated using scintillation counting in 4 ml Ultima Gold scintillating fluid. One unit of dCK was defined as the amount of enzyme catalysing the formation of 1 nmol dCMP/h at 37°C.

Statistical analysis

All determinations were made in duplicate using the same PBMC preparation. For statistical analysis, the following non-parametric tests were used: Mann–Whitney U test for comparison of median values and Spearman's rho test to establish the existence of correlation between different parameters. A P value of < 0.05 was considered significant. Analysis was performed using a standard statistical software (SPSS, version 11.0; Chicago, Illinois, USA).

To determine the intra-assay variability of TK and dCK assays, three samples were tested in five separate determinations. The coefficient of variation (CV) was 15% for TK activity and 4.2% for dCK activity. The intra-individual variability of TK and dCK activity was studied in four healthy donors whose blood was collected twice in 2 weeks. The CV was 18% for TK activity and 7.0% for dCK activity.

Results

TK activity in PBMC from HIV-positive and healthy donors

PBMC from 26 healthy donors and from 45 HIV-positive patients were examined for TK activity versus zidovudine and thymidine.

TK activity versus zidovudine

In healthy volunteers, the TK activity level ranged from 0.08 to 0.3 U/mg of protein, with a median value of 0.23 U (Fig. 1). The mean value for TK activity (± SD) in healthy volunteers was 0.21 ± 0.077 U/mg protein, with a CV of 36.6%. In HIV-infected individuals, the TK activity ranged from 0.08 to 2.0 U/mg protein, with a median value of 0.32 U (Fig. 1a). The mean value (± SD) of TK activity in these patients was 0.39 ± 0.34 U/mg protein, with a CV of 87.2%. Thus, a statistically significant increase was seen in the level of TK activity in HIV-infected individuals compared with healthy

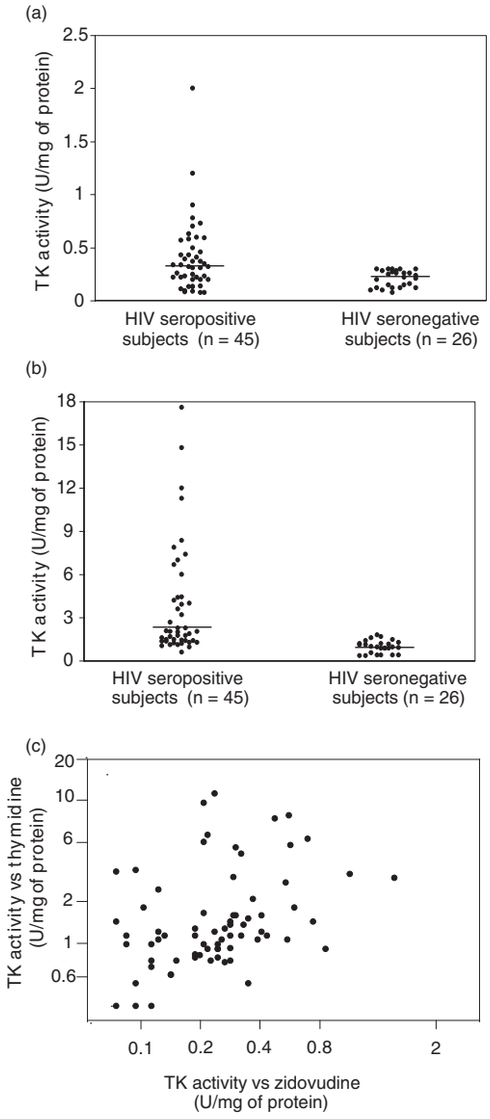

Fig. 1. (a) Levels of TK activity versus zidovudine in PBMC cells from HIV-seropositive patients and from healthy volunteers. High inter-individual variability in TK activity was found in HIV-infected individuals (CV, 87.2). There is a significant difference between the two groups ($P < 0.01$). Horizontal lines show the median of the data for each group. (b) Levels of TK activity versus deoxythymidine in PBMC from HIV-seropositive patients and from healthy volunteers. High inter-individual variability in TK activity was found in HIV-infected individuals (CV, >100). There is a significant difference between the two groups ($P < 0.01$). Horizontal lines show the median of the data for each group. (c) Correlation between TK activity and zidovudine and between TK activity and thymidine ($p < 0.01$ by Spearman's test).

volunteers ($P < 0.01$). The extent of the variability was higher in HIV-infected patients: CV, 87.2% in the HIV-seropositive group and 36.6% in the healthy volunteers.

TK activity versus thymidine

In healthy volunteers the TK activity ranged from 0.36 to 1.8 U/mg protein, with a median value of 0.95 U (Fig. 1b). The mean value (± SD) of TK activity was 1.01 ± 0.38 U/mg protein with a CV of 37.6%. In the 45 HIV-infected individuals, the TK activity ranged from 0.6 to 17.6 U/mg protein, with a median value of 2.0 U (Fig. 1b). The mean value (± SD) of TK activity was 3.79 ± 3.85 U/mg protein. The data again show a statistically significant increase in the level of TK activity in HIV-infected patients compared with healthy individuals ($P < 0.01$). Also, in this case the extent of inter-individual variability in TK activity in PBMC from HIV-infected patients is particularly high: CV > 100%. It is worth noting that, as expected, there is a significant direct correlation between TK activity versus thymidine and TK activity versus zidovudine ($P < 0.01$) (Fig. 1c).

In order to evaluate whether the level of TK activity in PBMC of HIV-infected patients correlated with the number of HIV RNA copies in plasma, or with the number of CD4 cells, a regression analysis was undertaken in which the values of TK activity, the viral load and CD4 cell count were considered. The analysis indicated that no correlation existed between the activity of the enzyme and viral load or CD4 T-cell count. Furthermore, the effect of some factors, such as age and sex of individuals, on TK activity levels was examined in all included individuals (i.e., both HIV-infected and healthy donor groups). The results indicate that these parameters had no influence on the level of TK activity (data not shown).

dCK activity in PBMC from HIV-positive and healthy donors

For dCK also, inter-individual variability was observed in the HIV-positive patients. In healthy donors, dCK activity ranged from 3.1 to 5.5 U/mg protein, with a median value of 4.13 U/mg protein (Fig. 2). The mean

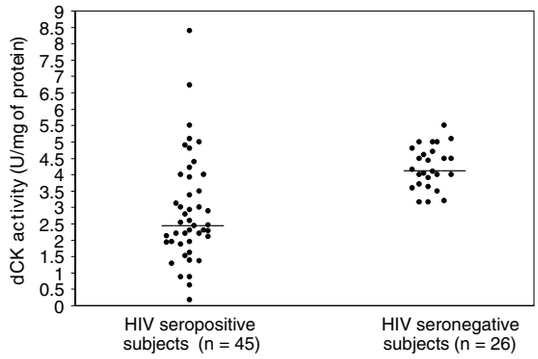

Fig. 2. Levels of dCK activity versus cytidine in PBMC from HIV-seropositive patients and from healthy volunteers. Inter-individual variability in dCK activity was found in HIV-infected individuals (CV, 55.8). There is a significant difference between the two groups ($P < 0.01$). Horizontal lines show the median of the data for each group.

value (± SD) of dCK activity was 4.22 ± 0.64 U/mg protein, with a CV of 15.1%. In HIV-positive patients, dCK activity levels ranged from 0.17 to 8.4 U/mg protein, with a median value of 2.46 U/mg protein (Fig. 2). The mean value (± SD) of dCK activity was 2.90 ± 1.62 U/mg protein, with a CV of 56%. The level of dCK activity is significantly reduced in HIV-infected patients compared with the enzymatic activity detected in healthy volunteers ($P < 0.01$).

For dCK there was no correlation between the level of enzymatic activity and HIV RNA or CD4 T cell count, age or sex of HIV-infected patients and healthy donors (data not shown).

Discussion

It is commonly considered that, at least to some extent, the response to antiretroviral treatment in HIV infection may be heterogeneous because of virological, immunological and pharmacological factors [1–3]. The results presented here provide further support for this. Our data indicate strongly that the degree of inter-individual variability in the activity of TK and, to a lesser extent, of dCK in PBMC from antiretroviral-naive, HIV-infected patients is much greater than that observed in healthy volunteers. Interestingly, the data show that each HIV-infected individual has a characteristic level of TK and dCK activity. This result was peculiar to the HIV-infected patients: no extensive variability was observed in the healthy volunteers. This lack of extensive variability in the activity of these enzymes in healthy volunteers combined with the lack of correlation between TK activity and parameters such as age or sex, may suggest that the variability has no genetic basis but, rather, depends on HIV infection. However, attempts to find a correlation with CD4 cell count or viral load level failed to demonstrate any significant correlation with the level of enzyme activity. Thus the determinants of such variability in HIV-infected individuals remain to be established and further studies are needed to explain this phenomenon. Nevertheless, in view of the role of enzymes in the metabolism of zidovudine and stavudine, the data strongly suggest that the activation of these drugs could vary considerably between individuals.

The data also show that TK activity is much higher in extracts from PBMC from seropositive individuals compared with those from healthy individuals. These findings suggest that HIV infection could up-regulate the activity of this cellular enzyme. Indeed, TK is cell cycle regulated, with a peak of activity at the S-phase, making it reasonable to assume that HIV infection, probably through induction of general activity in T-cell compartments, can induce an increase in TK activity. The increase in TK activity is consistent with data reported by Barry and colleagues [19], who observed that zidovudine is phosphorylated to a greater extent in HIV-positive patients than in healthy volunteers, with the findings of Wattanagoon and colleagues [20], who showed that the major metabolite in PBMC from HIV-infected patients is zidovudine monophosphate, and with the results of a study by Jorajuria and coworkers [21], where an increase of TK expression in PBMC from SIV-infected macaques was observed. In contrast our results do not appear to agree with those from an *in vitro* study published by Jacobsson and colleagues [22], in which PBMC from HIV-seropositive patients, stimulated in culture with a mitogen, had lower TK activity than PBMC from seronegative volunteers. Here, however, TK activity was evaluated in *ex vivo* experiments in which PBMC were not stimulated. Our data support the hypothesis previously formulated by Wattanagoon and colleagues [20] that although PBMC from HIV-positive patients have greater TK activity *in vivo*, their responsiveness to mitogen is decreased following culture [20,23].

On the basis of these considerations it can be hypothesized that HIV infection 'status' up-regulates *in vivo* TK activity. It is then possible to speculate that the reduction of TK activity reported by some authors [24–27] may be due not only to the direct effect of the drug on TK activity but also to the effect of the drug on viral load, which may, in turn, negatively modulate the TK activity. As stated before, although TK activity is increased in cells from HIV-infected individuals, this activity correlates neither with the plasma viral load nor with CD4 T-cell count. A possible explanation for this lack of correlation is that, in HIV-positive patients, several factors may contribute to the stimulation or the arresting of the cell cycle and, consequently, to the expression of TK. In fact, high levels of viraemia could indicate not only a high rate of virus replication, followed by a rapid turnover of CD4 T lymphocytes, but also the rapid death of infected cells. It is also known that some HIV products can modulate the phase of the cell cycle. Specifically, the *vpr* gene of HIV is known to encode a protein that prevents cell proliferation by causing arrest in the G_2/M phase of the cell cycle [28,29]. Since the amount of cytosolic TK increases in cells during transition from G_1 to S phase, we can speculate that the presence of large amounts of Vpr protein in cells may lead to a reduction in TK expression.

Although the type of the assay used to measure the enzymatic activities is a widely used and currently accepted method [30], it is important to note that because it is performed with cell extracts it is not possible to establish whether the variability and the increased enzymatic activity may be ascribed to cytosolic TK (TK1) and/or to mitochondrial TK (TK2). However, because the variability and increased TK activity are also seen when zidovudine (which is known to be preferentially phosphorylated by TK1 [30,31]) is used as substrate in the TK assay, these changes are probably

due to TK1. Based on current knowledge, we can exclude the possibility that such an assay can be used to measure the activity of enzymes other than TK that are eventually involved in the phosphorylation of zidovudine.

We have also analysed the activity of dCK in this study. Again, although to a lesser extent, a large inter-individual variability between HIV-seropositive patients was observed. This finding is consistent with the data reported by Moore and colleagues [32], who showed great variability in the intracellular pharmacokinetics of lamivudine phosphorylation in PBMC from asymptomatic HIV-positive patients. However, in contrast to what was observed for TK activity, HIV infection seemed to down-regulate the activity of this enzyme. A significant reduction in the mean value of dCK activity was observed in HIV-infected patients compared with the activity detected in PBMC from healthy volunteers. Thus, we can hypothesize that HIV infection affects dCK activity also. As dCK activity is not affected by the phase of the cell cycle [13,14], the reason for its decrement and variability in HIV-infected patients is less obvious. However, it is possible to speculate that the decreased activity of this enzyme could be caused by the effects of virus replication on cell viability and that different rates of replication may result in different levels of enzyme expression.

It should be mentioned that the observation of the decreased activity of dCK in HIV-positive patients is not consistent with the data reported by Wang *et al.,* who shows a trend of higher phosphorylation of emtricitabine, for which the first step of activation depends on dCK, in HIV-positive patients compared with healthy volunteers [33]. The data, however, are not directly comparable because here we are measuring the enzymatic activity of dCK, while Wang *et al*. measured the intracellular concentration of emtricitabine-TP, which is known to be the result of the activity of several cellular enzymes. It should be also mentioned that, as in the case of TK, the use of cell extracts does not imply that we are measuring dCK activity only. Indeed, dCyd is phosphorylated also by mitochondrial TK2. However, as this enzyme is present at lower levels than dCK in lymphocyte extracts [30] it is possible to infer that the enzymatic activity detected in our experiments is mainly due to dCK. Further studies are needed to address this important and intriguing issue.

In conclusion, together our results indicate that TK and dCK are differently expressed in HIV-infected patients and that their enzymatic activities in HIV-infected patients differ from those in healthy volunteers.

Because of the existence of a different (from TK) rate-limiting step in the phosphorylation pathway of zidovudine, it remains to be established whether the observed differences and the variability of expression of these enzymes may affect the rate of ddNTP formation and, ultimately, whether the above findings have clinical significance. However, data indirectly indicating that the above variability could affect the response to antiretroviral therapy do exist. Indeed, Groschel and colleagues [34] reported a positive correlation between TK activity and CD4 cell count in patients undergoing antiretroviral therapy, while the activity of this enzyme negatively correlated with viral load. The same authors suggested that these observations imply that differing levels of TK activity might lead to differences in the amounts of ddNTP, which could in turn result in different responses to therapy. As our patients were not treated, this issue was not addressed; nevertheless our data indirectly indicate that: Phase I clinical trials, which are usually conducted in healthy volunteers, may not give accurate information on the metabolism in HIV-positive patients of drugs that require the activity of these enzymes; the first step of drug phosphorylation may be different for HIV-positive patients treated with drugs such as zidovudine, stavudine or lamivudine that depend on the TK and dCK enzymes for their activation.

In our opinion, an explanation of the viral or cellular factors that contribute to this variability, as well as their effect on the response to antiretroviral treatment, may help to optimize the management of HIV patients.

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References

1. Yeni PG, Hammer SM, Carpenter CC, Cooper DA, Fischl MA, Gatell JM, *et al*. **Antiretroviral treatment for adult HIV infection in 2002: updated recommendations of the International AIDS Society-USA Panel.** *JAMA* 2002; **288**:222–335.
2. Fletcher CV. **Pharmacological consideration for therapeutic success with antiretroviral agents.** *Ann Pharmacother* 1999; **33**:989–995.
3. Hirsch MS, Brun-Vezinet F, Clotet B, Conway B, Kuritzkes DR, D'Aquila RT, *et al*. **Antiretroviral drug resistance testing in adults infected with human immunodeficiency virus type 1: 2003 recommendations of an International AIDS Society-USA Panel.** *Clin Infect Dis* 2003; **37**:113–128.
4. Holford N, Sheiner L. **Understanding the dose-effect relationship: clinical application of pharmacokinetic-pharmacodynamic models.** *Clin Pharmacokinet* 1981; **6**:429–453.
5. Back DJ, Gatti G, Fletcher C, Garaffo R, Haubrich R, Hoetelmans R, *et al*. **Therapeutic drug monitoring in HIV infection: current status and future directions.** *AIDS* 2002; **16**:S5–S37.
6. Gerber JG, Acosta EP. **Therapeutic drug monitoring in the treatment of HIV-infection.** *J Clin Virol* 2003; **27**:117–128.
7. Back DJ, Khoo SH. **The role of clinical pharmacology in optimizing antiretroviral therapy.** *J Clin Pharmacol* 2003; **55**:473–476.
8. Burger D, Hugen P, Reiss P, Gyssen I, Schneider M, Kroon F, *et al*. **Therapeutic drug monitoring of nelfinavir and indinavir in treatment-naive HIV-1 infected individuals.** *AIDS* 2003; **17**:1157–1165.

9. Fletcher CV, Acosta EP, Henry K, Page LM, Gross CR, Kawel SP, *et al*. **Concentration-controlled zidovudine therapy.** *Clin Pharmacol Ther* 1998; **64**:331–338.
10. Fletcher CV, Anderson PL, Kakuda TN, Schacker TW, Henry K, Gross CR. **Concentration controlled compare with conventional antiretroviral therapy for HIV infection.** *AIDS* 2002; **16**:551–560.
11. Fletcher CV, Kawel SP, Kakuda TN, Anderson PL, Weller D, Bushman LR, *et al*. **Zidovudine triphosphate and lamivudine triphosphate concentration-response relationship in HIV-infected persons.** *AIDS* 2000; **14**:2137–2144.
12. Hoggard PG, Sales SD, Kewn S, Sunderland D, Khoo SH, Hart CA, *et al*. **Correlation between intracellular pharmacological activation of nucleoside analogues and HIV suppression *in vitro*.** *Antivir Chem Chemother* 2000; **11**:353–358.
13. Gao WY, Shirasaka T, Johns DG, Broder S, Mitzuya H. **Differential phosphorylation of azidothymidine, dideoxycytidine and dideoxyinosine in resting and activated peripheral blood mononuclear cells.** *J Clin Invest* 1993; **91**:2326–2333.
14. Gao WY, Agbaria R, Driscoll JS, Mitsuya H. **Divergent anti-human immunodeficiency virus activity and anabolic phosphorylation of 2′,3′-dideoxynucleoside analogs in resting and activated human cells.** *J Biol Chem* 1994; **269**:12633–12638.
15. Shirasaka T, Chokekijchai S, Yamada A, Gosselin G, Imbach JL, Mitsuya H. **Comparative analysis of anti human immunodeficiency virus type 1 activities of dideoxynucleoside analogs in resting and activated peripheral blood mononuclear cells.** *Antimicrob Agents Chemother* 1995; **39**:2555–2559.
16. Piliero PJ. **Pharmacokinetic properties of nucleoside/nucleotide reverse transcriptase inhibitors.** *J Acquir Immune Defic Syndr* 2004; **37**:S2–S12.
17. Balzarini J, Heredewijn P, De Clerq E. **Differential patterns of intracellular metabolism of 2′,3′-dideoxy-2′,3′-didehydrothymidine and 3′-azido-2′,3′-dideoxythymidine, two potent anti-human immunodeficiency virus compounds.** *J Biol Chem* 1989; **284**:6127–6133.
18. Maga G, Verri A, Bonizzi L, Ponti G, Poli G, Garbesi A, *et al*. **Lack of stereospecificity of suid pseudorabies virus thymidine kinase.** *Biochem J* 1993; **294**:381–385.
19. Barry M, Wild M, Veal G, Back D, Breckenridge A, Fox R, *et al*. **Zidovudine phosphorylation in HIV-infected patients and seronegative volunteers.** *AIDS* 1994; **8**:F1–F5.
20. Wattanagoon Y, Bangchang KN, Hoggard PG, Khoo SH, Gibbons SE, Khoo SH, *et al*. **Pharmacokinetics of zidovudine phosphorylation in human immunodeficiency virus-positive Thai patients and healthy volunteers.** *Antimicrob Agents Chemother* 2000; **44**:1986–1989.
21. Jorajuria S, Clayette P, Dereuddre-Bosquet N, Benlhassan-Chahour K, Thiébot H, Vaslin B, *et al*. **The expression of P-glycoprotein and cellular kinase is modulated at the transcriptional level by infection and highly active antiretroviral therapy in a primate model of AIDS.** *AIDS Res Hum Retrovir* 2003; **19**:307–311.
22. Jacobsson B, Britton S, Tornevik Y, Eriksson S. **Decrease in thymidylate kinase activity in peripheral blood mononuclear cells from HIV-infected individuals.** *Biochem Pharmacol* 1998; **56**:389–395.
23. Clerici M, Stocks NI, Zajac RA, Boswell RN, Lucey DR, Via CS, *et al*. **Detection of three distinct patterns of T helper cell dysfunction in asymptomatic human immunodeficiency virus-seropositive patients. Independence of CD4+ cell numbers and clinical staging.** *J Clin Invest* 1989; **84**:1892–1899.
24. Avramis VI, Kwock R, Solorzano MM, Gomperts E. **Evidence of *in vitro* development of drug resistance to azidothymidine in T-lymphocytic leukemia cell line (Jurkat E6-1/AZT-100) and in pediatric patients with HIV-1 infection.** *J Acquir Immune Defic Syndr* 1993; **6**:1287–1296.
25. Antonelli G, Turriziani O, Verri A, Narciso P, Ferri F, D'Offizi G, *et al*. **Long-term exposure to zidovudine affects *in vitro* and *in vivo* the efficiency of phosphorylation of thymidine kinase.** *AIDS Res Hum Retrovir* 1996; **12**:223–228.
26. Groschel B, Hover G, Doerr HW, Cinatl J Jr. **Zidovudine (AZT) resistance in H9 cells due to decreased TK expression is associated with hypermetylation of TK gene.** *Nucleosides Nucleotides Nucleic Acid* 2001; **20**:487–492.
27. Turriziani O, Scagnolari C, Bambacioni F, Bellomi F, Focher F, Gentile M, *et al*. **Selection of a T-cell line resistant to stavudine and zidovudine by prolonged treatment with stavudine.** *Antiviral Ther* 2002; **7**:105–111.
28. Chowdhury IH, Wang XF, Landau NR, Robb ML, Polonis VR, Birx DL, *et al*. **HIV-1 Vpr activates cell cycle inhibitors p21/waf/cip1: a potential mechanism of G2/M cell cycle arrest.** *Virology* 2003; **305**:371–377.
29. Janket ML, Manickam P, Majumder B, Thotala D, Wagner M, Schafer EA, *et al*. **Differential regulation of host cellular genes by HIV-1 viral protein R (vpr): cDNA microarray analysis using isogenic virus.** *Biochem Biophys Res Commun* 2004; **314**:1126–1132.
30. Arner ES, Spasokoukotskaja T, Erikson 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* 1992; **188**:712–718.
31. Muench-Petersen B, Cloos L, Tyrsted G, Eriksson S. **Diverging substrate specificity of human thymidine kinase 1 and 2 against antiviral dideoxynucleosides.** *J Biol Chem* 1991; **266**:9032–9038.
32. Moore KHP, Barrett JE, Shaw S, Pakes GE, Churchus R, Kapoor A, *et al*. **The pharmacokinetics of lamivudine phosphorylation in peripheral blood mononuclear cells from patients infected with HIV-1.** *AIDS* 1999; **13**:2239–2250.
33. Wang LH, Begley J, St.Claire RL, Harris J, Wakeford C, Rousseau FS. **Pharmacokinetic and pharmacodynamic characteristics of emtricitabine support its once daily dosing for the treatment of HIV infection.** *AIDS Res Hum Retroviruses* 2004; **20**:1173–1182.
34. Groschel B, Miller V, Doerr HW, Cinatl J Jr. **Activity of cellular thymidine kinase 1 in PBMC of HIV-1 infected patients: novel therapy marker.** *Infection* 2000; **28**:209–213.