Biochemical Pharmacology, Vol. 54, pp. 979–990, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00253-0

Intracellular Metabolism of 3′-Azido-3′-deoxythymidine (AZT): A Nuclear Magnetic Resonance Study on T-lymphoblastoid Cell Lines with Different Resistance to AZT

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ABSTRACT. This paper reports the results of ^{31}P and ^{1}H nuclear magnetic resonance (NMR) studies on the uptake and phosphorylation of 3′-azido-3′-deoxythymidine (AZT) in the human CD4$^+$ T-lymphoblastoid cell line CCRF-CEM (CEM-1.3) and in its AZT-resistant cell variant MT-500, isolated by prolonged culturing of CEM cells in the presence of increasing AZT concentrations. After 3 hr of incubation in the presence of 0.5 mM AZT, both AZT and its monophosphorylated form (AZT-MP) could be detected in the sensitive cell line in concentrations above the NMR detection levels. In another cell line, MOLT-4, which is less sensitive to AZT effects, the intracellular level of AZT-MP was much lower and was only slightly raised by increasing the concentration of AZT in the extracellular and intracellular compartments. In the AZT-resistant clone MT-500, characterized by a very low thymidine kinase (TK, EC 2.7.1.21) activity with respect to the parental clone, the intracellular AZT-MP concentration was below detection (<0.02 nmol/10^6 cells). Since, however, not only AZT-MP but also AZT signals failed to be detected in MT-500 extracts following cell incubation with AZT, it was concluded that a TK deficiency cannot be the exclusive mechanism of AZT resistance in these cells. The possible effects of additional mechanisms of drug resistance, such as specific AZT cell extrusion and limited permeation, are discussed, together with the new prospects offered by NMR spectroscopy to further evaluate the limiting steps for the utilization of antiretroviral nucleoside analogues. BIOCHEM PHARMACOL **54**;9:979–990, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. AZT; MDR; AZT resistance; thymidine kinase; pyrimidine 5′-nucleotidase; NMR; lymphoid cells

The activity of a number of nucleoside analogues against the human immunodeficiency virus (HIV)** is now well recognized [1–5]. Several reports state that these drugs, upon entering the infected cells either passively via nonfacilitated diffusion [6] or actively through specific nucleobase transporters [7], are efficiently converted into their monophosphorylated derivatives by host nucleoside kinases and subsequently, to a lower extent, to di- and triphosphates by nucleotide kinases [8, 9]. The latter compounds are believed to be the active forms that interrupt the viral DNA chain elongation [3, 8, 10–13], but a major role has also been ascribed to both the nucleoside and its monophosphorylated form in the cytostatic and cytotoxic effects exerted by some of these drugs on infected cells [14, 15]. In contrast, there is no acute toxicity toward healthy uninfected cells because of the relative insensitivity of eukaryotic polymerases, a characteristic that strongly favored the use of these analogues in anti-HIV therapy [8, 10, 16].

Serious problems may, however, arise if the infected cells develop, upon prolonged therapy with these drugs, membrane transport systems specifically devoted to actively extrude the drug before its conversion to the active form(s). This phenomenon, well known under the general name of multidrug resistance (MDR) [17, 18] is often responsible for therapeutic failure in human cancer treatment. It seems

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** *Abbreviations:* AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-3′-deoxythymidine; AZT-MP, 3′-azido-3′-deoxythymidine 5′-monophosphate; CEM-1.3, T-lymphoblastoid cell line CCRF-CEM; DSS, 2,2-dimethyl-2-silanepentane-5-sulfonate; FCS, fetal calf serum; Glx, glutamic acid and glutamine; HIV, human immunodeficiency virus; MDPA, methylenediphosphonic acid; MDR, multidrug resistance; PCho, phosphocholine; NMR, nuclear magnetic resonance; TK, thymidine kinase; UDPHex, uridine diphosphohexose.

Received 26 November 1996; accepted 11 March 1997.

plausible that HIV-infected cells may also utilize similar membrane pumps or more specific ones to decrease the intracellular concentration of antiretroviral compounds [19, 20] and their phosphorylated derivatives.

On these grounds, it seems particularly interesting to assess the intracellular concentration levels of anti-HIV nucleoside and nucleotide analogues in human lymphoid cells to evaluate the efficiency of the enzymatic steps responsible for drug phosphorylation, as well as the metabolic alteration(s) involved in cell resistance to these therapeutic agents.

This paper presents the results of nuclear magnetic resonance (NMR) studies on the metabolic fate of the most commonly used anti-HIV drug, 3′-azido-3′-deoxythymidine (AZT), in human T-lymphoblastoid cell lines cultivated *in vitro*. Despite its relatively low sensitivity [21–23], multinuclear NMR has been extensively applied in recent years to investigate the metabolic pathways through which some drugs, usually labeled with NMR-detectable isotopes, undergo bioactivation or degradation processes [24–26]. A peculiar advantage of this spectroscopic approach is the simultaneous detection and monitoring of several metabolites, with no further separation or derivatization steps as are usually required by conventional methods such as HPLC, GC, and liquid scintillation counting of radiolabeled compounds [27, 28]. Moreover, the parallel detection in the NMR spectra of resonances attributable to low molecular weight metabolites, such as ATP, ADP, phospholipid derivatives, and amino acids [21–23, 29], may provide additional information on cell biochemistry and therefore on possible side effects exerted by the drug and its derivatives on the metabolic state of the target cell.

^{31}P and ^{1}H NMR analyses carried out in this study allowed us to identify and monitor specific signals arising from both AZT and its monophosphorylated form (AZT-MP), the di- and triphosphorylated AZT derivatives (AZT-DP and AZT-TP) remaining undetectable because of the low sensitivity inherent to NMR spectroscopy.

Two lymphoblastoid cell lines were selected in this study as AZT targets, MOLT-4 and CCRF-CEM (CEM-1.3), both derived from human $CD4^+$ T cells [30, 31] and therefore possible specific targets of HIV infection in the acquired immunodeficiency syndrome (AIDS). The intracellular AZT metabolism was investigated in uninfected cells. It has in fact been reported that in terms of biochemical conversion and pharmacological efficacy, AZT behaves in the same manner in either uninfected or HIV-infected cells [32]. It is also worth mentioning that the inhibitory effects of AZT on cell growth are reported to be quite different in these two cell lines, CEM-1.3 being more prone to the AZT cytostatic effect [9, 33]. Because of their reported lower sensitivity to AZT, MOLT-4 cells were utilized in our work as further controls constitutively expressing an intermediate level of resistance.

On the other hand, similarly to other lymphoblastoid cell lines [34], a prolonged exposure of CEM-1.3 to progressively increasing concentrations of AZT has been shown to induce the expression of AZT-specific cellular resistance not involving an enhanced expression of the P-glycoprotein [35]. A decreased activity (10–20-fold) of thymidine kinase (TK) has been demonstrated in these drug-resistant cells [34, 35]. This enzyme, responsible for the conversion of AZT into AZT-MP, represents an essential step for the further activation of the drug to AZT-TP. The decreased activity of TK in AZT-resistant cells has been interpreted on the basis of an AZT-induced hypermethylation, resulting in a decreased expression of the TK gene [36–38]. Therefore, a prolonged exposure to the drug may in principle select cell variants expressing alternative (or combined) mechanisms to avoid or reduce AZT-induced harmful side effects: 1) altered permeation of AZT across the cell membrane; 2) drug extrusion by efflux systems; 3) reduced AZT phosphorylation; and 4) increased nucleotidase-mediated AZT-MP hydrolysis. These mechanisms are expected to exert different effects on AZT and AZT-MP levels in either AZT-sensitive or in its resistant cell variant (Table 1). This study investigates the potentialities offered by NMR spectroscopy for a possible elucidation of the mechanism(s) responsible for the onset of AZT resistance in human lymphoid cells.

TABLE 1. Possible mechanisms of cell resistance to AZT in MT-500 (r) vs. CEM-1.3 (s) cells

Intracellular drug levels	Biochemical mechanisms
$[AZT\text{-}MP(r)] \ll [AZT\text{-}MP(s)]$	a) Limited permeation in resistant cells (at equilibrium)
and	*or*
$[AZT(r)] \ll [AZT(s)]$	b) Drug extrusion from resistant cells
$[AZT\text{-}MP(r)] \ll [AZT\text{-}MP(s)]$	c) Reduced thymidine kinase activity in resistant cells
but	*or*
$[AZT(r)] \geq [AZT(s)]$	d) Increased pyrimidine-5′-nucleotidase activity in resistant cells

MATERIALS AND METHODS

Chemicals

AZT, AZT-MP, HEPES, EDTA, [2-^{14}C]CMP, 40–60 mCi/mmol, [2-^{14}C]AZT-MP, 40–60 mCi/mmol, and deuterium oxide (D_2O, 99.9 atom % D) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methylenediphosphonic acid (MDPA) and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) were from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals were of the highest purity available. RPMI-1640 and fetal calf serum (FCS) were purchased from Gibco (Grand Island, NY, USA).

Cells

For this study the human T-lymphoblastoid $CD4^+$ cell lines MOLT-4 [30] and CEM-1.3 [31] were used, together with

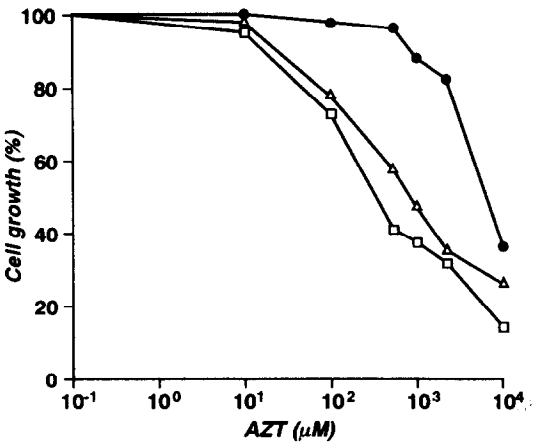

FIG. 1. Growth rate assay of CEM-1.3, MT-500, and MOLT-4 cell lines after 48 hr in the presence of increasing AZT concentrations. The data shown, determined by a single typical experiment repeated three times with similar results, are expressed as percent of control. □, CEM-1.3; ●, MT-500; △, MOLT-4.

the AZT-resistant cell variant MT-500 [35] isolated by prolonged exposure of CEM-1.3 to increasing drug concentration (up to 500 μg/mL). An extensive phenotypic characterization of MT-500 demonstrated that the expression of cellular determinants, including CD4, was not altered in this cell line.* These cell lines were cultivated in FCS-enriched RPMI-1640. During cell growth, the AZT-resistant strain was maintained either in the absence (MT-500[−]) or in the presence (MT-500[+]) of 0.5 mM AZT, a treatment that failed to induce any significant alteration in either cell morphology or viability in this variant. Figure 1 illustrates the different levels of sensitivity of these cell lines to AZT. After the 72-hr growth period, cells were washed with 0.9% NaCl and centrifuged for 5 min at 400 × g; the resulting pellet was resuspended in RPMI-1640 without FCS. Only some experiments performed with MOLT-4 required the presence of 2% FCS. The incubation media contained AZT concentrations ranging from 0.5 to 2 mM, and the exposure time varied between 1 and 6 hr.

Pyrimidine-5'-Nucleotidase Activity (EC 3.1.3.5)

The enzymatic assay on the different cell lines was performed according to Beutler [39]. Briefly, 60 μL of cell lysates (obtained from 50 × 10^6 cells in 0.5 mL of 10 mM HEPES/0.5 mM EDTA, pH 7.2) were incubated for 30 min at 37° with 40 μL of a reaction mixture composed of 10 mM $MgCl_2$ and either 0.75 mM CMP (40–60 mCi/mmol) or AZT-MP (40–60 mCi/mmol). The reaction was stopped by addition of 0.2 mL of 0.15 M $Ba(OH)_2$ followed by 0.2 mL of $ZnSO_4$ and 0.5 mL of water. After mixing well, the tubes were centrifuged for 10 min at 10,000 × g, and then 0.5-mL aliquots of the supernatant solution were counted in 5 mL of scintillation fluid Aquasol-2 (Amersham, Arlington Heights, IL, USA). The blank was obtained for each sample by carrying out the $Ba(OH)_2$-$ZnSO_4$ precipitation immediately after adding cell lysates to the reaction mixture. The total ^{14}C activity of the labeled substrate was measured by adding 0.96 mL of H_2O to 40 μL of the premixed reaction mixture and counting 0.5 mL in 5 mL of the scintillation fluid. The enzyme activity (E) was calculated as milliunits (number of nanomoles of CMP or AZT-MP hydrolyzed per minute) per gram of total protein, according to Beutler [39].

Preparation of Cell Samples for NMR Analysis

At selected time intervals, cell incubation with the drug was stopped by 5 min of centrifugation at 400 × g. The pelleted cells were resuspended in drug-free medium and centrifuged again. The cell pellet, which contained approximately 2 to 3 × 10^8 cells, was treated with cold ethanol according to a previously described procedure [40]. Briefly, about 2 × 10^8 cells were added to 5 mL of a cold 70% (v/v) ethanol/water solution. The resulting mixture was then sonicated for six time intervals of 30 sec each at maximum power to achieve complete disruption of cell membranes. Upon standing overnight at −20°, the ethanolic cell extracts were dried in a rotary evaporator (Rotovapor-R, Büchi, Switzerland) at an outside temperature of 25–30°, corresponding to an internal temperature of the ethanolic-water sample during evaporation of 5–10°. The dried extracts were stored at −20°.

NMR Spectroscopy

For ^{31}P NMR analyses, the dried samples were redissolved in 3 mL of a D_2O solution containing 20 mM HEPES (pH 7.2), 50 mM EDTA, and 0.5 mM MDPA (as internal NMR standard) and then transferred into 10-mm O.D. NMR tubes. Analyses were performed at 25° on a Bruker AMX 400 spectrometer (Bruker Analytische Messtechnik GmbH, Karlsruhe, Germany) working at a frequency of 161.98 MHz. Each spectrum was obtained by accumulating 8–16 × 10^3 scans with a 3.4-sec cycling time and a pulse width corresponding to a magnetization flip angle of 60°.

For ^1H NMR analyses, the dried samples were resuspended in 500 μL of D_2O and placed in 5-mm O.D. NMR tubes. The analyses were carried out at 25° by a Varian GEMINI 200 spectrometer (Varian Associates, Inc., Palo Alto, CA, USA) set at a frequency of 199.98 MHz. The spectra were obtained by accumulating a number of scans ranging between 256 and 1024, with a cycling time of 5.9 sec and a pulse width corresponding to a 60° flip angle. The residual HDO signal was suppressed by selective irradiation with a presaturating continuous wave power. The chemical shift scale of proton spectra was determined by using DSS as internal standard.

Peak assignments were performed either by comparison with data reported in the literature or by utilizing solutions

* Cianfriglia M, unpublished observations.

of selected standard compounds. In some cases the reference molecules were directly added to the cell extract before the NMR analysis. Intracellular concentrations of AZT and AZT-MP were determined in cell extracts upon addition of known amounts of the respective authentic standard compounds to the sample as well as by comparing the relevant peak integrals with those of standard mixtures of known composition.

RESULTS

^{31}P NMR analyses performed on MOLT-4 cells incubated at 37° in the presence of 2 mM AZT (Fig. 2A) demonstrated a significant accumulation of AZT-MP, detected in cell extracts as a single pH-dependent resonance and identified by adding the authentic compound to the extract. After 3 hr of cell incubation, the area of this peak corresponded to an intracellular AZT-MP concentration of about 0.4 nmol/10^6 cells (Table 2). The intensity of the signal arising from the β-phosphate of ATP in AZT-treated cells (Fig. 2A) was not significantly different from that of untreated cells (Fig. 2B) indicating that, despite the rather high concentration of AZT used, the energy charge of these cells was not affected to any substantial extent. Incubation times of 3 hr were chosen, since the signal intensity was highest at that point, remaining practically constant at longer times.

CEM-1.3 cells, more prone than MOLT-4 to the cytotoxic effects of AZT, were incubated in the presence of a lower concentration of the drug (0.5 mM instead of 2 mM). In fact, incubation with 2 mM AZT caused a marked loss of viability in CEM-1.3 cells, as demonstrated by the trypan-blue exclusion test (data not shown). After incubation of these cells in the presence of AZT, a substantial amount of AZT-MP was detected in the sensitive strain (Fig. 3A). Extracts of the AZT-resistant cells failed, instead, to exhibit any signal arising from AZT-MP (Fig. 3B). The presence or absence of AZT in the medium during the 72-hr period of cell growth preceding the actual experiment did not increase AZT-MP above detectability levels in AZT-resistant cells upon 3 hr of incubation with 0.5 mM AZT. It should also be noted that there were no major differences between the CEM-1.3 and MT-500 cells in terms of other signals arising from phospholipid metabolites such as phosphocholine (PCho) and from ATP and ADP, either before (not shown) or after (Fig. 3A, B) cell incubation with AZT. Enzymatic assays were carried out to assess whether an increased 5′-nucleotidase activity could be responsible for the lack of a detectable AZT-MP signal in MT-500 cell extracts after cell incubation with AZT. These biochemical analyses showed that the activity of this enzyme was actually very similar in both the CEM-1.3 and MT-500 cell lines (Fig. 4). Moreover, AZT-MP appeared to be a poor substrate for this enzyme (as compared with its true substrate, CMP) in all the investigated cell systems (Fig. 4).

^1H NMR spectroscopy analyses of cell extracts allowed the simultaneous measurement of intracellular AZT and AZT-MP concentration levels in both MOLT-4 and CEM-1.3 cells after their incubation with AZT. In fact, preliminary observations on ^1H spectra obtained from a standard mixture of AZT and AZT-MP dissolved in D_2O led to the identification of different resonances due to nonexchangeable protons covalently linked to either the AZT ribosyl ring or the pyrimidine base (Fig. 5). There was a clear distinction of the signals respectively arising from the nonphosphorylated and monophosphorylated forms of the drug. In particular, all AZT-MP resonances, with the sole exception of H2′ protons, showed a low field shift as compared with the signals of the corresponding AZT chemical groups (Fig. 5 and Table 3). The highest resolution was observed at the level of the H5′ proton multiplet, centered at 3.843 ppm in AZT and at 4.114 ppm in AZT-MP, the difference in chemical shift likely being due to the proximity to the phosphoryl group (Fig. 5). Unfortunately, the H5′ signal as well as the resonances arising from H4′ and H3′ of the ribosyl ring, were of little value in analyses of cell extracts, due to their position in a most crowded region of their spectra. Better information was obtained from the two singlets positioned at 7.660 and 1.897 ppm (for AZT) and at 7.776 and 1.924 ppm (for AZT-MP) attributable to the 6-heterocyclic (H6) and 5-methyl protons (CH_3(C5)), respectively (Fig. 5). This assignment is supported by the appearance of the signals in the aromatic or aliphatic spectral regions, respectively, as well as on the basis of the ratio between their peak areas. Also noteworthy are the triplets centered at 6.230 (AZT) and 6.286 ppm (AZT-MP), respectively, ascribed to the anomeric H1′ proton of the ribose moiety, characteristically split into three subcomponents because of coupling with the protons on the 2′-deoxy carbon atom. These signals could be rather easily detected among the others of typical ^1H spectra of ethanolic extracts of either MOLT-4 or CEM-1.3 cells incubated with AZT (Fig. 6A and B), with a further check being made with the same cells in the absence of the drug (not shown). The most useful resonances for quantification of AZT and AZT-MP in cell extracts were: a) the signal arising from CH_3(C5), detected at about 1.89 for AZT and 1.93 for AZT-MP, at the high-field side of the Hβ protons of glutamic acid and glutamine (Glx); b) the signal assigned to the H6 proton of the thymine heterocyclic ring, approximately 7.66 for AZT and 7.85 for AZT-MP on the high-field side of the analogous protons of UTP and uridine diphosphohexose (UDPHex). The signal from the H1′ proton of the ribose moiety of AZT, located at about 6.23 ppm in MOLT-4 as well as in the pure standard (on the low-field side of the H1′ protons of ATP and ADP) had an unexpected downfield shift in CEM-1.3, where it could not be resolved from the analogous signal of AZT-MP. The analysis of both H6 and CH_3(C5) ^1H NMR signals in ethanolic extracts of cells preincubated for 3 hr with AZT showed a [AZT]/[AZT-MP] ratio of 1.5:1.0 in MOLT-4 cells and 1.0:3.5 in CEM-1.3 (upon incubation with 0.5 mM AZT, Table 2). The same conditions of exposure to the drug failed to induce any

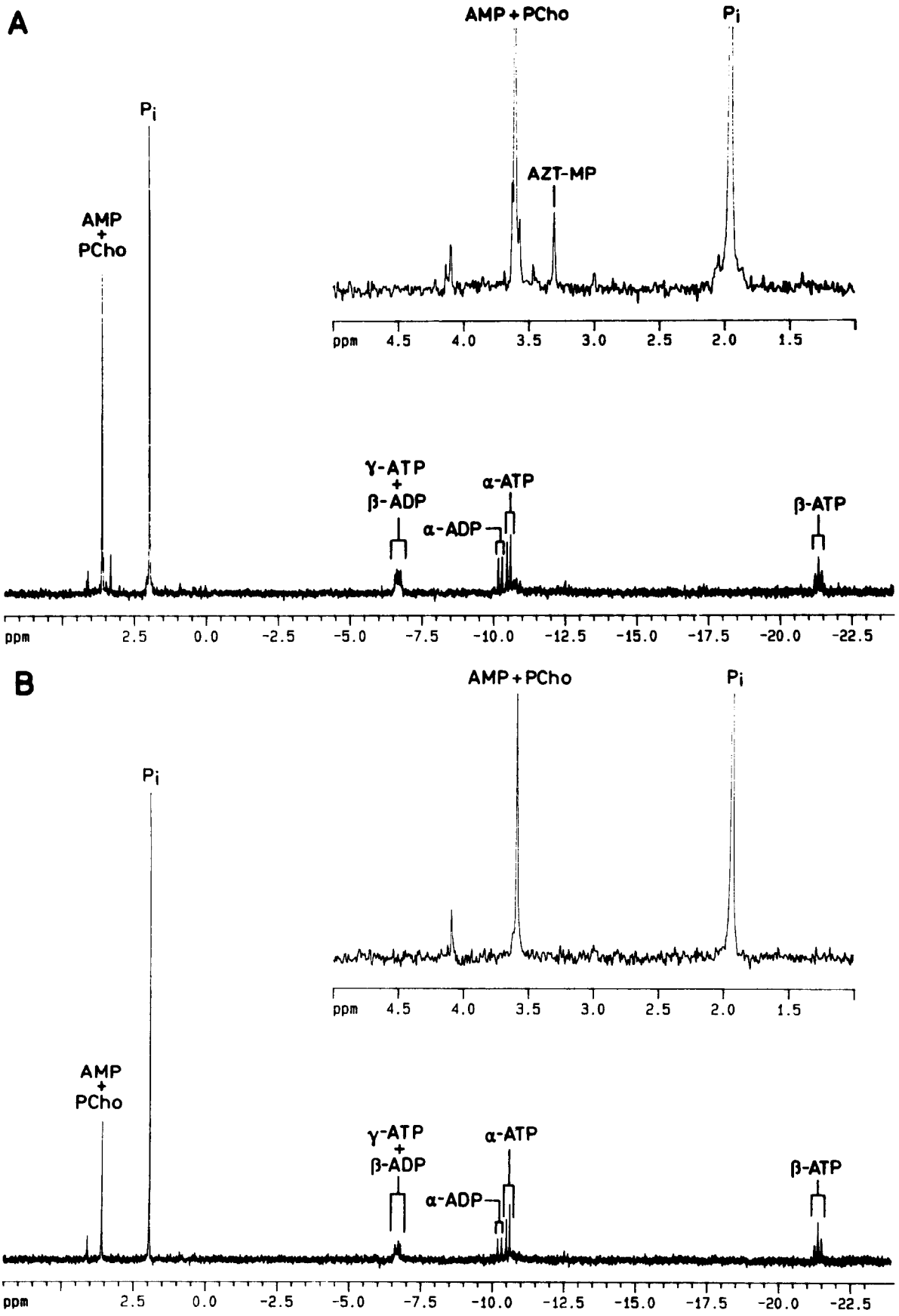

FIG. 2. ^{31}P NMR spectra (161.9 MHz) of ethanolic extracts prepared from MOLT-4 cells incubated for 3 hr in the presence (A) or absence (B) of 2 mM AZT. P_i, inorganic phosphate.

TABLE 2. Intracellular concentration of AZT-MP (nmol/10^6 cells) detected by means of NMR spectroscopy in ethanolic extracts of lymphoblastoid cell lines incubated for 3 hr in the presence of AZT

Cell line	Extracellular AZT (mM)	Intracellular AZT-MP (^{31}P NMR)*	Intracellular AZT (^{1}H NMR)*	Intracellular AZT-MP (^{1}H NMR)*	[AZT]/:[AZT-MP]†
MOLT4	2.0	0.4	2.3	0.5	4.5:1.0
	0.5	n.d.	0.5	0.3	1.5:1.0
CEM-1.3	0.5	1.7 ± 0.6‡	0.5	1.7 ± 0.5‡	1.0:3.5
MT-500	0.5	<0.15	<0.02	<0.02	n.d.

n.d., not determined.
* nmol/10^6 cells.
† From ^{1}H NMR spectra.
‡ Average ± SD (n = 4).

detectable appearance of either AZT or AZT-MP signals in the extracts of MT-500 cells (Fig. 7). These results, together with those of ^{31}P NMR analyses (Fig. 3), indicate that not only AZT-MP but also AZT were under NMR detectability levels in AZT-resistant cells.

DISCUSSION

Among the nucleoside analogues presently utilized as antiretroviral drugs, the thymidine analogue AZT has up to now held a preeminent position in the treatment of the acquired immunodeficiency syndrome. Two major hindrances, however, constrain the clinical handiness and efficacy of this drug. The first arises from an increasing incidence of HIV isolates that do not incorporate the analogue into their genome, as well as from a decreased capability exhibited by some target cells (namely lymphocytes) in terms of cellular uptake and/or subsequent phosphorylation of AZT. The second limitation is due to the onset, in the course of the therapeutic treatment, of toxic side effects such as bone marrow cell suppression and delayed myopathy, which can appear at high as well as low dosage levels [41–43].

A main purpose of this study was to assess the potentialities offered by NMR spectroscopy in measuring the intracellular levels of AZT (both in its nonphosphorylated and phosphorylated forms) in different human lymphoblastoid cell lines, either sensitive or specifically resistant to this antiretroviral agent. Our data show that MOLT-4 cells, grown in the absence of AZT before drug treatment, are able to accumulate AZT-MP above the ^{31}P NMR detection level (Fig. 2A and Table 2). This finding is further

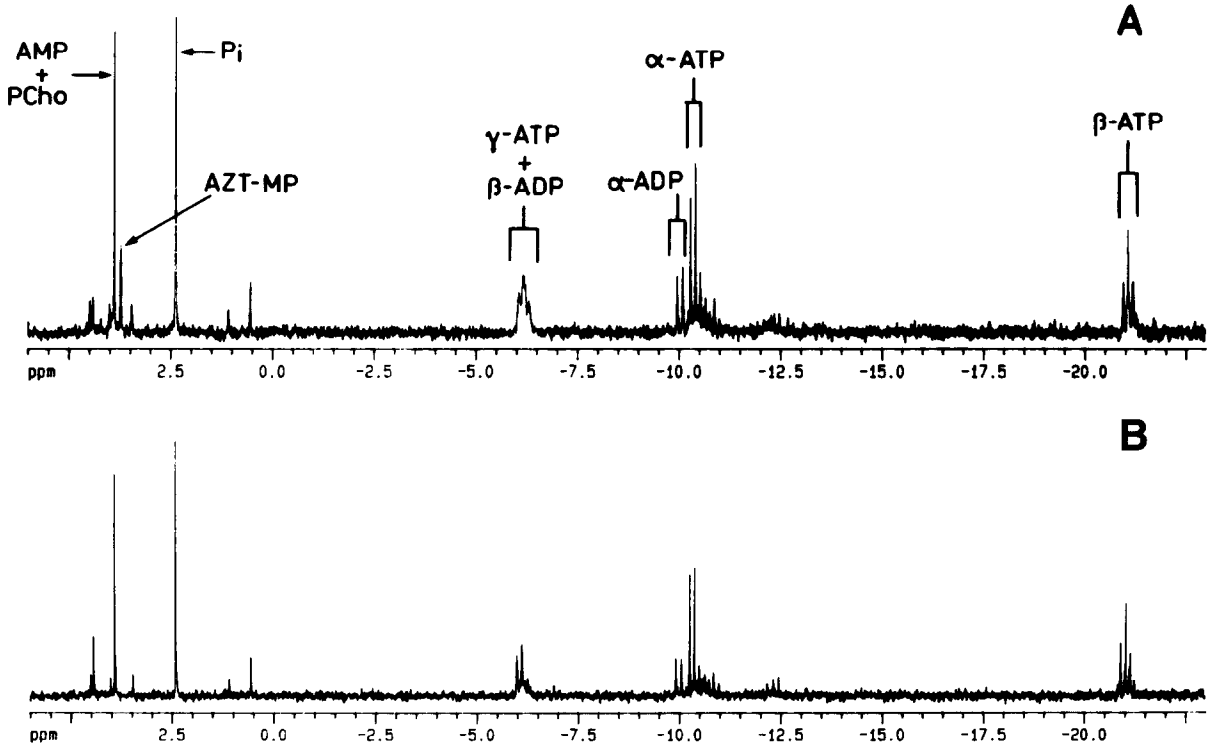

FIG. 3. ^{31}P NMR spectra of ethanolic extracts prepared from CEM-1.3 (A) and MT-500 (B) cell lines after 3 hr of incubation in the presence of 0.5 mM AZT.

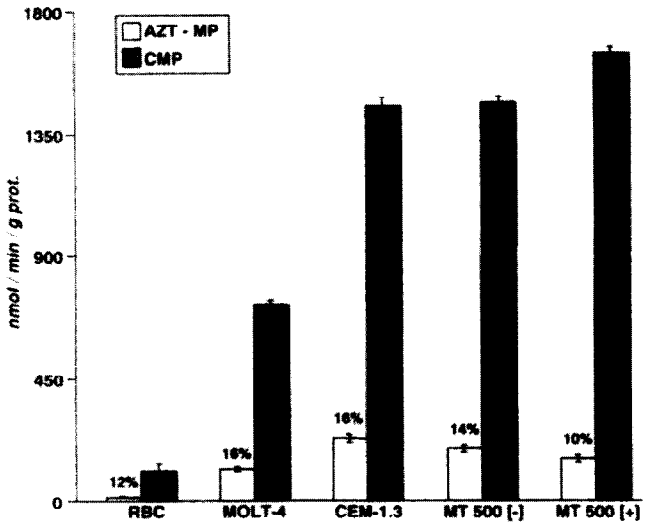

FIG. 4. Pyrimidine-5′-nucleotidase activity measured, utilizing as substrate either CMP (gray column) or AZT-MP (white columns), in the following human cell types: a) erythrocytes (RBC); b) MOLT-4; c) CEM-1.3; d) MT-500[−] cultivated for 72 hr with no AZT in the growth medium; and e) MT-500[+] cultivated in the presence of 0.5 mM AZT. See "Materials and Methods" for further details. The figures above the white columns represent the percent activity on AZT-MP as compared with CMP in the same cells.

supported by the identification of distinct ^{1}H NMR signals in cell extracts, attributed to the unphosphorylated compound and to its mononucleotide derivative (Fig. 6A). These observations confirm previous reports in the literature [27] according to which AZT penetrates into the cell, where it is phosphorylated by a nucleoside cellular kinase to AZT-MP before undergoing further phosphorylation steps mediated by nucleotide kinases. Di- and triphosphonucleotides do not accumulate in the cell to NMR-detectable concentration levels. ^{31}P NMR analyses also showed that even after a rather prolonged exposure (3 hr) to high AZT levels (2 mM), MOLT-4 cells were still able to maintain practically unaltered ATP pools (Fig. 2). Moreover, analyses of ^{1}H NMR spectra of MOLT-4 cell extracts showed that, while a fourfold rise in external AZT corresponded to a fourfold increase in the intracellular AZT pool, AZT-MP increased less than twofold, indicating that in these cells TK activity, rather than AZT uptake, is the limiting step for utilization of the drug. In the experiments carried out on CEM-1.3 cells, a lower AZT concentration had to be selected for their treatment (0.5 mM instead of 2 mM) because these cells were more prone to the cytotoxic effects of this antiretroviral agent. The AZT-MP concentration ranges measured by NMR in MOLT-4 and CEM-1.3 cell extracts after a 3-hr exposure to AZT were basically consistent with those obtained in previous studies [8, 44], where AZT-MP levels ranging between 0.5 and 2.4 nmol/10^6 cells were reported after longer exposures (up to 24 hr) of H9 and CCRF-CEM cells to 0.025 mM AZT.

There was, however, a significant difference in the balance of drug uptake and phosphorylation detected by ^{1}H NMR in the two investigated AZT-sensitive lines, MOLT-4 and CEM-1.3. In fact, under the same conditions of exposure to the drug (a 3-hr incubation with 0.5 mM AZT), the [AZT]:[AZT-MP] ratio was approximately 1.5:1 in MOLT-4 and 1:3.5 in CEM-1.3 cells, while the intracellular level of AZT was practically the same (0.5 nmol/10^6 cells). The observed difference in the [AZT]:[AZT-MP] ratio, likely due to a different TK activity in the two AZT-sensitive cell lines, may represent one of the major causes of the variability observed in the response of different cell lines to AZT [8, 9]. The difference observed in AZT phosphorylation between MOLT-4 and CEM-1.3 cells (Table 2) may even reflect a more general phenomenon capable of explaining the reduction or even the lack of efficacy of this drug in some individuals or in different cells and tissues of the same subject. In particular, the MOLT-4 cell line, which constitutively exhibits lower sensitivity to AZT, might be envisaged as a partially "AZT-resistant" strain. This constitutively expressed lower sensitivity of MOLT-4 cells to AZT can be attributed to their reduced AZT-phosphorylating ability, probably through selection of less active enzyme(s) or a down-regulation of TK gene expression. The acquired, AZT-induced, specific resistance of MT-500 cells is instead characterized by the absence of NMR-detectable intracellular levels of both AZT and AZT-MP, suggesting a mechanism whereby AZT permeation, and not only AZT phosphorylation, is the limiting step in regulating the sensitivity of these cells to the drug.

Another important source of cell unresponsiveness to AZT could be the triggering, upon prolonged cell exposure to the compound, of mechanisms responsible for a progressive drug resistance that finally abolishes its therapeutic efficacy [4]. In addition to the well known mechanism of drug extrusion by membrane-associated glycoproteins, enzymatic or metabolic alterations may also contribute to these phenomena; it thus appeared interesting to compare the NMR spectral features of CEM-1.3 with those of its derived AZT-resistant strain, MT-500. These studies demonstrated that the ATP/ADP levels were not significantly different in the two strains, suggesting that the biochemical patterns related to the energetic state were probably not significantly altered in the drug-resistant phenotype.

^{31}P NMR spectra of CEM-1.3 and MT-500 cell extracts demonstrated a clearcut difference at the level of AZT metabolism in these cell lines following cell exposure to AZT (Fig. 3). The same pattern was observed in ^{1}H NMR spectra (Fig. 6B and Fig. 7). In fact, while, as discussed above, a 3-hr cell incubation in the presence of 0.5 mM AZT induced an average accumulation of AZT-MP of 1.7 nmol/10^6 cells in the sensitive CEM-1.3 cell line, no AZT-MP signal could be detected in ^{31}P NMR spectra of MT-500 cell extracts. It is worth emphasizing that the absence of this signal in NMR spectra of MT-500 cell extracts does not necessarily mean that no AZT-MP is formed in these cells but only that its steady-state concentration is below detection levels of both ^{31}P and ^{1}H NMR. In agreement with estimates in the literature [22], the

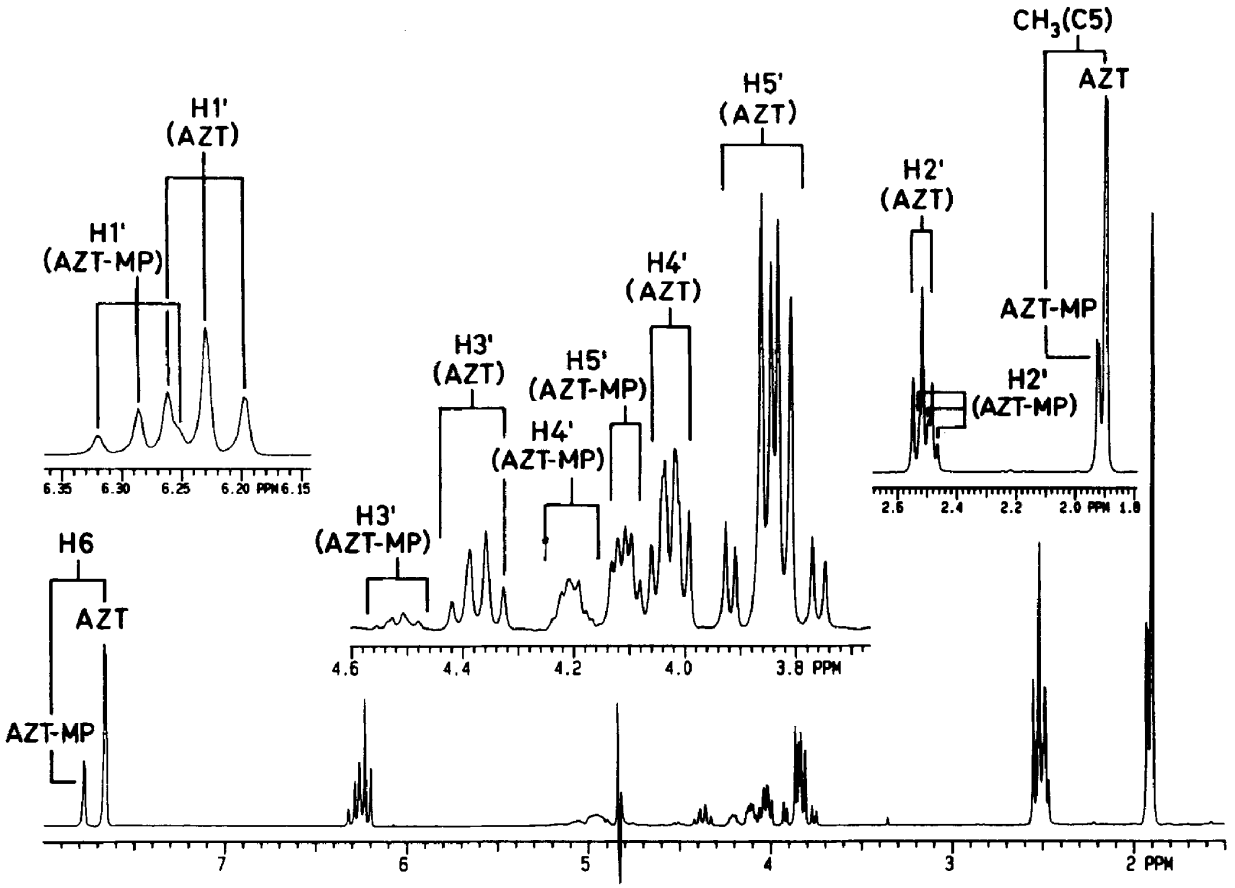

FIG. 5. ^{1}H NMR spectrum of a mixture of AZT-MP (2.9 mM) and AZT (6.7 mM) in D_2O. See "Results" for further details.

sensitivity threshold of our ^{31}P NMR spectra was approximately 0.01 mM, corresponding to a detectability level of AZT-MP in cell extracts of approximately 0.15 nmol/10^6 cells, i.e. about 10-fold lower than that measured in CEM-1.3 extracts. The detection threshold was further decreased to about 0.02 nmol/10^6 cells in ^{1}H NMR spectra of cell extracts, indicating that the accumulation of AZT-MP in resistant MT-500 cells was at least 80 ± 30 times lower than that found in the parental AZT-sensitive line. Dianzani *et al.* [35] had already shown that the rate of accumulation of the [^{3}H]AZT pool in these cells was about 60-fold lower with respect to that of the parental CEM-1.3 strain, AZT-MP being, according to these authors, the main component of this intracellular pool. A similar reduction (up to 100-fold) in AZT-MP content has also been reported, starting from the AZT-sensitive Jurkat E6-1 T cell to the increasingly more resistant Jurkat E6-1/AZT-20, -50, and -100 cell lines [34].

The decrease under detectability levels of AZT-MP signals in NMR spectra of extracts of AZT-resistant MT-500 cells could not be ascribed to an increase in the rate of AZT-MP degradation mediated by pyrimidine 5′-nucleotidase (mechanism d, Table 1). In fact, no significant difference was observed in the activity of this enzyme in AZT-resistant cells with respect to the parental AZT-sensitive cell line (Fig. 4) in assays performed by using

TABLE 3. Peak assignment in ^{1}H NMR spectra of a mixture of AZT-MP and AZT

Chemical group	Chemical shift (ppm)					
	AZT			AZT-MP		
	Mixture*	MOLT-4†	CEM-1.3†	Mixture*	MOLT-4†	CEM-1.3†
C_3(C5)	1.897(s)	1.886(s)	1.900(s)	1.924(s)	1.925(s)	1.940(s)
H5′	3.843	n.d.	n.d.	4.114	n.d.	n.d.
H1′	6.230(*t*)‡	6.224(*t*)‡	6.293(*t*)‡	6.286(*t*)‡	6.278(*t*)‡	6.293(*t*)‡
H6	7.660	7.652	7.665	7.776	7.842	7.855

n.d., not detected.
* Mixture of standard AZT and AZT-MP.
† Ethanolic extracts (See "Materials and Methods").
‡ Center of multiplet; s, singlet; *t*, triplet.

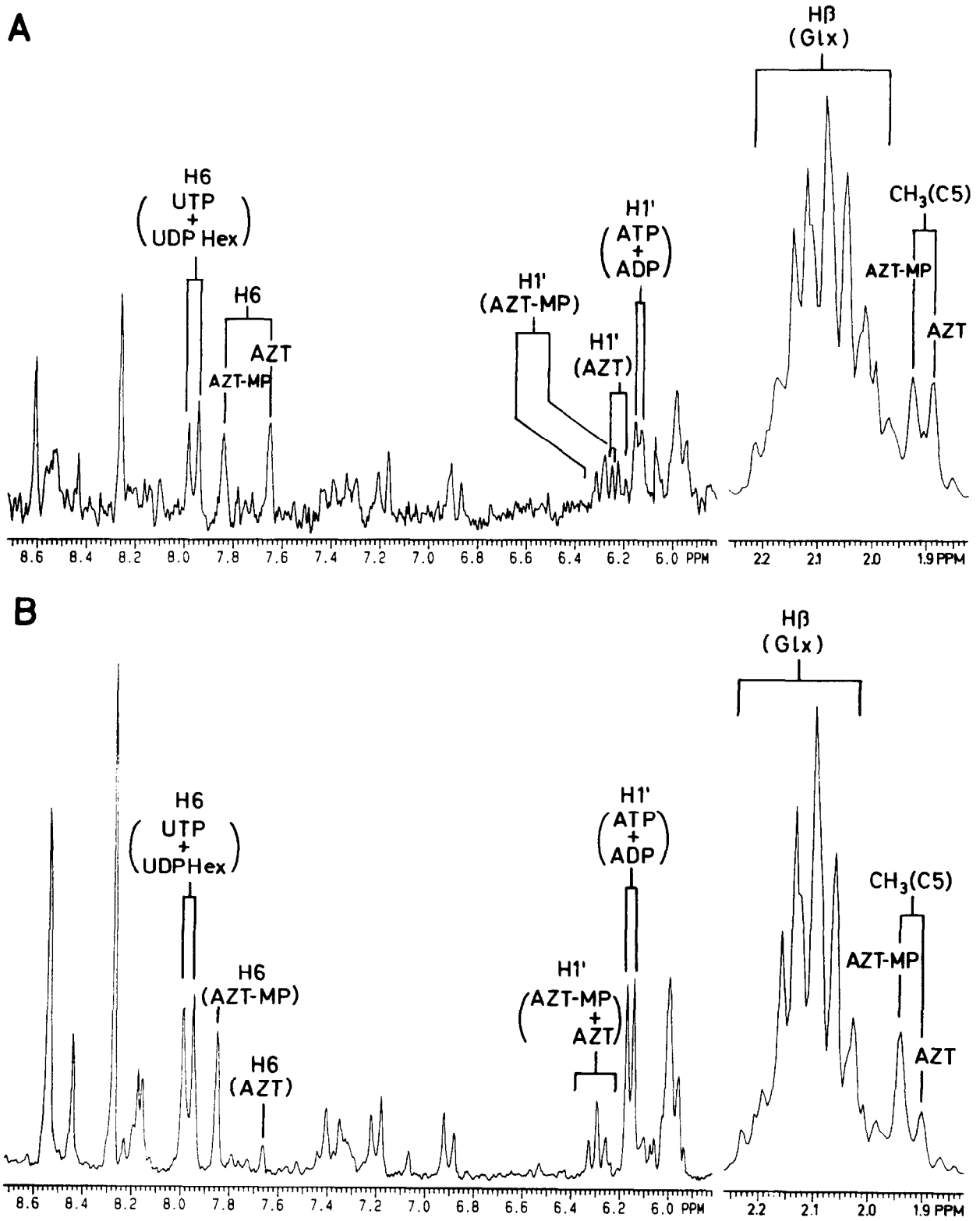

FIG. 6. ^{1}H NMR spectra of ethanolic extracts prepared from cells incubated for 3 hr in the presence of 0.5 mM AZT (A, MOLT-4; B, CEM-1.3). Two expanded spectral regions are shown, selected from the aromatic side of the spectrum between 5.8 and 8.7 ppm (left) and from the aliphatic side between 1.82 and 2.26 ppm (right), respectively. The vertical scale of the aromatic side is fourfold that of the aliphatic side. See "Results" for further details.

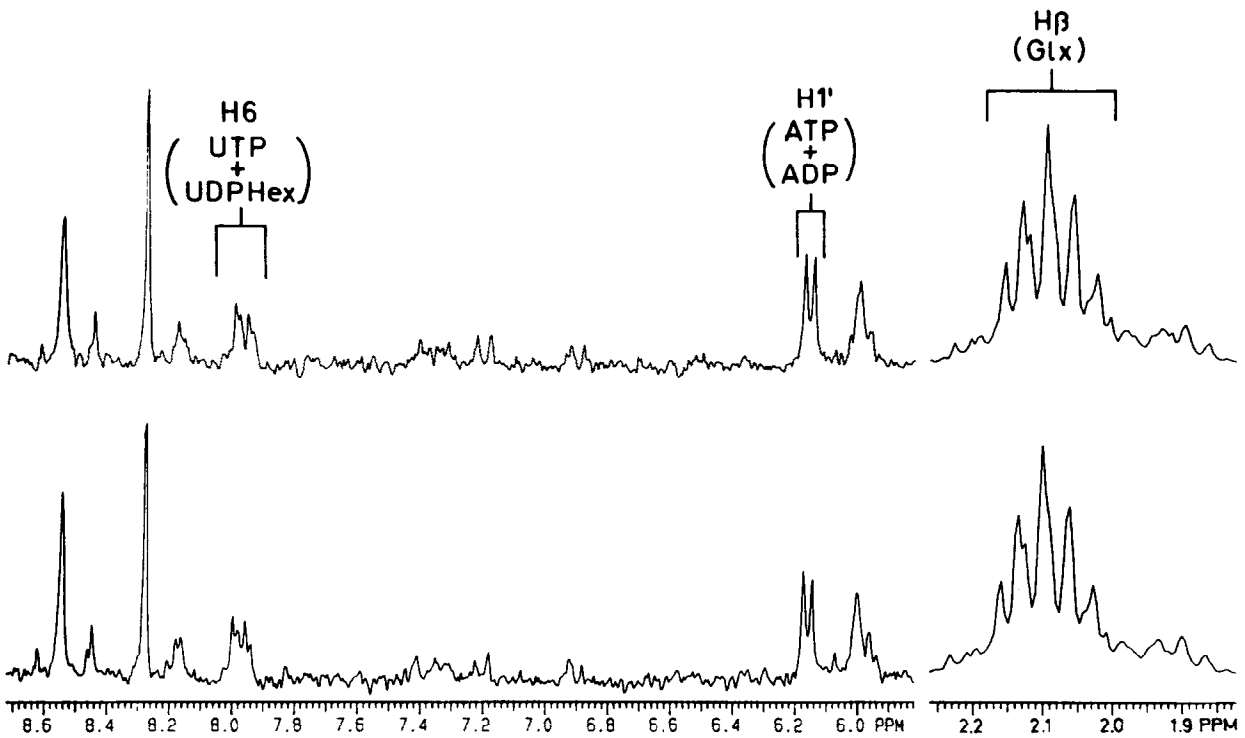

FIG. 7. ^{1}H NMR spectra obtained from ethanolic extracts of MT-500 cells after 3 hr of incubation in the presence (upper trace) or in the absence (lower trace) of 0.5 mM AZT. Expanded spectral regions are as in Fig. 6.

either AZT-MP or the natural reaction substrate, CMP. It is noteworthy that more significant differences were actually detected by comparing the pyrimidine-5′-nucleotidase activity levels measured in either the CEM-1.3 or MT-500 strains with those found in MOLT-4 cells (approximately twofold lower) and in human erythrocytes (about 16-fold lower). These observations indicate an apparent lack of correlation of this enzymatic activity with AZT-induced cellular resistance. Moreover, the lower efficiency always displayed by AZT-MP with respect to CMP, as substrate of pyrimidine-5′-nucleotidase, assigns this enzyme a minor role, if any, in the catabolic pathway(s) involved in the intracellular clearance of AZT. The NMR spectra of MT-500 cell extracts, examined in the light of the other mechanisms of cell resistance (a, b, c) outlined in Table 1, suggest that these AZT-resistant cells likely possess, with respect to the parental sensitive cell line, not only a reduced ability to synthetize the mononucleotide by TK-mediated phosphorylation of AZT, but a drug extrusion capability as well. Although no direct experiments were performed to test the limited permeation hypothesis, this mechanism is unlikely in view of the strong dependence in the sensitive cells of intracellular AZT levels from the external AZT concentration.

A decreased TK activity, down to about 13% of the value exhibited by the Jurkat E6-1 sensitive strain, has been reported by Avramis *et al.* in the progressively more resistant Jurkat E6-1/AZT-10, -20, -50, and -100 cell lines [34]. An even more marked decrease in TK activity, down to 1–4% of the values found in the sensitive parental strains, has also been observed in CEM/BrdUrd and in $CEM_{AZT2000}$ cells by Tornevik *et al.* [14] and by Dianzani *et al.* [35], respectively. It may also be noted that such a marked reduction in TK activity levels is still likely to ensure a sufficient supply of phosphorylated thymidine to these cells, thanks to the much higher affinity exhibited by this enzyme for its physiological substrate. In fact, it has been reported that the K_m values of TK activity toward thymidine are generally in the range of 3–7 μM [34], a concentration level below that observed during the usual cell growth conditions (20–30 μM).

A reduced TK activity cannot, however, be the only mechanism responsible for the strong decrease in AZT-MP levels in the AZT-resistant cells, otherwise higher intracellular AZT levels would be observed in MT-500 than in CEM-1.3 cells. Since this is not the case, as demonstrated by the lack of any detectable AZT signal in either ^{31}P or ^{1}H NMR spectra of ethanolic extracts of MT-500 cells, additional mechanisms are likely to contribute to the AZT-resistant phenotype exhibited by these cells. We recently found an increased hypermethylation of exon 1 of the TK gene both in CEM 1.3 and, more markedly, in MT-500 cells exposed to AZT, even in the absence of a more generalized DNA hypermethylation [38]. This phenomenon could be the molecular basis for the transcriptional inactivation of the TK gene. Moreover, although the MT-500 cells are negative for the glycoprotein, a transient positivity has been observed during the selection procedure.* The possibility afforded by NMR spectroscopy to simultaneously detect intracellular AZT and AZT-MP should be exploited

to evaluate the existence of AZT-specific extrusion mechanisms and their temperature and energy dependence. Similar analyses should be performed with other antiviral nucleoside analogues. Finally, the balance between restriction of intracellular AZT availability and AZT-MP formation could be modified by transfecting the MT-500 cells with a viral TK gene.

We thank Dr. Pei Jin Zhang for participation in NMR measurements and M. Giannini and M. Giambenedetti for excellent technical assistance in the maintenance of NMR equipment. This work was supported in part by CNR Grant 95.02152 (A.B.), CNR and MURST 40% "Proqetti Nazionali" (R.S.), and an AIDS Research Contract from ISS, Italy (M.C., C.S.). A.F. was a Fellow of the Italian Research Program on AIDS from 1992 to 1994.

References

1. Mitsuya H and Broder S, Strategies for antiviral therapy in AIDS. *Nature* **325:** 773–778, 1987.
2. Herdewijn P, Balzarini J, De Clercq E, Pauwels R, Baba M, Broder S and Vanderhaeghe H, 3′-substituted 2′,3′-dideoxynucleoside analogues as potential anti-HIV (HTLV-III/LAV) agents. *J Med Chem* **30:** 1270–1278, 1987.
3. Mitsuya H, Jarett RF, Matsukura M, Veronese FDM, De Vico AL, Sarngadharan MG, Johns DG, Reitz MS and Broder S, Long-term inhibition of human T-lymphotropic virus type III/lymphadenopathy-associated virus (human immunodeficiency virus) DNA synthesis and RNA expression in T cells protected by 2′,3′-dideoxynucleosides *in vitro*. *Proc Natl Acad Sci USA* **84:** 2033–2037, 1987.
4. Fischl MA, Richman DD, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Schooley RT, Jackson GG, Durack DT, King D and the AZT Collaborative Working 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.
5. Yarchoan R, Mitsuya H, Myers CE and Broder S, Clinical pharmacology of 3′-azido-2′,3′-dideoxythymidine (zidovudine) and related dideoxynucleosides. *N Engl J Med* **321:** 726–738, 1989.
6. Zimmerman TP, Mahony WB and Prus KL, 3′-Azido-3′-deoxythymidine: an unusual nucleoside analog that permeates the membrane of human erythrocytes and lymphocytes by nonfacilitated diffusion. *J Biol Chem* **262:** 5748–5754, 1987.
7. Domin BA, Mahony WB and Zimmerman TP, Purine nucleobase transport in human erythrocytes: reinvestigation with a novel "inhibitor-stop" assay. *J Biol Chem* **263:** 9276–9284, 1988.
8. Furman PA, Fyfe JA, St. Clair MH, Weinhold K, Rideout JL, Freeman GA, Nusinoff Lehrman S, 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.
9. Balzarini J, Pauwels R, Baba M, Herdewijn P, De Clercq E, Broder S and Johns DG, The *in vitro* and *in vivo* antiretrovirus activity, and intracellular metabolism of 3′-azido-2′,3′-dideoxythymidine and 2′,3′-dideoxycytidine are highly dependent on the cell species. *Biochem Pharmacol* **37:** 897–903, 1988.
10. Waqar MA, Evans MJ, Manly KF, Hughes RG and Huberman JA, Effects of 2′,3′-dideoxynucleosides on mammalian cells and viruses. *J Cell Physiol* **121:** 402–408, 1984.
11. Cheng YC, Dutschman GE, Bastow KF, Sarngadharan MG and Ting RY, Human immunodeficiency virus reverse transcriptase: general properties and its interactions with nucleoside triphosphate analogs. *J Biol Chem* **262:** 2187–2189, 1987.
12. Vrang L, Bazin H, Remaud G, Chattopadhyaya J and Oberg B, Inhibition of the reverse transcriptase from HIV by 3′-azido-3′-deoxythymidine triphosphate and its *threo* analogue. *Antiviral Res* **7:** 139–149, 1987.
13. Kedar PS, Abbots J, Kovacs T, Lesiak K, Torrence W and Wilson SH, Mechanism of HIV reverse transcriptase: enzyme-primer interaction as revealed through studies of a dNTP analogue, 3′-azido-dTTP. *Biochemistry* **29:** 3603–3611, 1990.
14. Tornevik Y, Ullman B, Balzarini J, Wahren B and Eriksson S, Cytotoxicity of 3′-azido-3′-deoxythymidine correlates with 3′-azidothymidine-5′-monophosphate (AZTMP) levels, whereas anti-human immunodeficiency virus (HIV) activity correlates with 3′-azidothymidine-5′-triphosphate (AZTTP) levels in cultured CEM T-lymphoblastoid cells. *Biochem Pharmacol* **49:** 829–837, 1995.
15. Cretton EM, Xie MY, Bevan RJ, Goudgaon NM, Schinazi RF and Sommadossi JP, Catabolism of 3′-azido-3′-deoxythymidine in hepatocytes and liver microsomes, with evidence of formation of 3′-amino-3′-deoxythymidine, a highly toxic catabolite for human bone marrow cells. *Mol Pharmacol* **39:** 258–266, 1991.
16. 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.
17. Bradley G, Juranka PF and Ling V, Mechanism of multidrug resistance. *Biochim Biophys Acta* **948:** 87–128, 1988.
18. Endicott JA and Ling V, The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu Rev Biochem* **58:** 137–171, 1989.
19. Yusa K, Oh-hara T, Yamazaki A, Tsukahara S, Satoh W and Tsuruo T, Cross-resistance to anti-HIV nucleoside analogues in multidrug-resistant human cell. *Biochem Biophys Res Commun* **169:** 986–990, 1990.
20. Antonelli G, Turriziani O, Cianfriglia M, Riva E, Dong G, Fattorossi A and Dianzani F, Resistance of HIV-1 to AZT might also involve the cellular expression of multidrug resistance P-glycoprotein. *AIDS Res Hum Retroviruses* **8:** 1839–1843, 1992.
21. Gadian DG, *Nuclear Magnetic Resonance and its Applications to Living Systems*. Clarendon Press, Oxford, 1982.
22. De Certaines JD, Bovée WMMJ and Podo F (Eds.) *Magnetic Resonance Spectroscopy in Biology and Medicine. Functional and Pathological Tissue Characterization*. Pergamon Press, Oxford, 1992.
23. Sze DY and Jardetzky O, Determination of metabolite and nucleotide concentrations in proliferating lymphocytes by ^{1}H-NMR of acid extracts. *Biochim Biophys Acta* **1054:** 181–197, 1990.
24. Prior MJW, Maxwell RJ and Griffiths JR, *In vivo* ^{19}F-NMR spectroscopy of the antimetabolite 5-fluorouracil and its analogues. *Biochem Pharmacol* **39:** 857–863, 1990.
25. Malet-Martino MC and Martino R, Uses and limitations of nuclear magnetic resonance (NMR) spectroscopy in clinical pharmacokinetics. *Clin Pharmacokinet* **20:** 337–349, 1991.
26. Newell DR, Maxwell RJ and Golding BT, *In vivo* and *ex vivo* magnetic resonance spectroscopy as applied to pharmacokinetic studies with anticancer agents: a review. *NMR Biomed* **5:** 273–278, 1992.
27. Toyoshima T, Kimura S, Muramatsu S, Takahagi H and Shimada K, A sensitive nonisotopic method for the determination of intracellular azidothymidine 5′-mono-, 5′-di-, and 5′-triphospate. *Anal Biochem* **196:** 302–307, 1991.
28. Singhal RP, Hughbanks D and Xian J, Separation of dideoxyribonucleosides in trace amounts by automated liquid

chromatography and capillary electrophoresis. *J Chromatogr* **609:** 147–161, 1992.
29. Berners-Price SJ, Sant ME, Christopherson RI and Kuchel PW, ^{1}H and ^{31}P NMR and HPLC studies of mouse L1210 leukemia cell extracts: the effect of Au(I) and Cu(I) diphosphine complexes on the cell metabolism. *Magn Reson Med* **18:** 142–158, 1991.
30. Minowada J, Ohnuma T and Moore GE, Rosette-forming human lymphoid cell lines: establishment and evidence for origin of thymus-derived lymphocytes. *J Natl Cancer Inst* **49:** 891–895, 1972.
31. Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA and McCarthy RE, Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer* **18:** 522–529, 1965.
32. Singlas E, Pioger JC, Taburet AM, Colaneri S and Fillastre JP, Comparative pharmacokinetics of zidovudine (AZT) and its metabolite (G.AZT) in healthy subjects and HIV seropositive patients. *Eur J Clin Pharmacol* **36:** 639–640, 1989.
33. Avramis VI, Markson W, Jackson RL and Gomperts E, Biochemical pharmacology of zidovudine in human T-lymphoblastoid cells (CEM). *AIDS* **3:** 417–422, 1989.
34. 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 AIDS* **6:** 1287–1296, 1993.
35. Dianzani F, Antonelli G, Turriziani O, Riva E, Simeoni E, Signoretti C, Strosselli S and Cianfriglia M, Zidovudine induces the expression of cellular resistance affecting its antiviral activity. *AIDS Res Hum Retroviruses* **10:** 1471–1478, 1994.
36. Nyce J, Leonard S, Canupp D, Schulz S and Wong S, Epigenetic mechanisms of drug resistance: drug-induced DNA hypermethylation and drug resistance. *Proc Natl Acad Sci USA* **90:** 2960–2964, 1993.
37. Wu S, Liu X, Solorzano MM, Kwock R and Avramis VI, 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 Acquir Immune Defic Syndr Human Retrovirol* **8:** 1–9, 1995.
38. Lucarelli M, Palitti F, Carotti D, Cianfriglia M, Signoretti C, Bozzi A and Strom R, AZT-induced hypermethylation of human thymidine kinase gene in the absence of total DNA hypermethylation. *FEBS Lett* **396:** 323–326, 1996.
39. Beutler E, Pyrimidine 5′-nucleotidase. In: *Red Cell Metabolism: a Manual of Biochemical Methods*. Ed. Beutler E, pp. 100–102. Grune & Stratton Inc., New York, 1983.
40. Ferretti A, Chen LL, Di Vito M, Barca S, Tombesi M, Cianfriglia M, Bozzi A, Strom R and Podo F, Pentose phosphate pathway alterations in multidrug resistant leukemic T-cells: ^{31}P NMR and enzymatic studies. *Anticancer Res* **13:** 867–872, 1993.
41. Gill PS, Rarick M, Brynes RK, Causey D, Loureiro C and Levine AM, Azidothymidine associated with bone marrow failure in the acquired immunodeficiency syndrome (AIDS). *Ann Intern Med* **107:** 502–505, 1987.
42. Richman DD, Fischl MA, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Hirsh MS, Jackson GG, Durack DT, Phil D, Nusinoff-Lehrman S and the AZT Collaborative Working Group, The toxicity 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:** 192–197, 1987.
43. Dalakas MC, Illa I, Pezeshkpour GH, Laukaitis JP, Cohen B and Griffin JL, Mitochondrial myopathy caused by long-term zidovudine therapy. *N Engl J Med* **322:** 1098–1105, 1990.
44. Fridland A, Connelly MC and Ashmun R, Relationship of deoxynucleotide changes to inhibition of DNA synthesis induced by the antiretroviral agent 3′-azido-3′-deoxythymidine and release of its monophosphate by human lymphoid cells (CCRF-CEM). *Mol Pharmacol* **37:** 665–670, 1990.