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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

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Abstract—Activation of the anti-human immunodeficiency virus (HIV) compound 3′-azido-3′-deoxythymidine (AZT) is dependent on its 5′-phosphorylation by cellular nucleoside and nucleotide kinases. Azidothymidine 5′-triphosphate (AZTTP) is considered to be the metabolite responsible for both the anti-HIV effect of AZT, via inhibition of reverse transcriptase, and cytoxicity by interference with cellular DNA polymerases. During the characterization of AZT metabolism in cultured human T-lymphoblastoid CEM cells, a spontaneously occurring variant cell line, CEM/Ag-1, was found that showed approximately 10-fold resistance to AZT growth inhibition as compared to wild type (wt) cells (EC_{50} = 2 mM as compared to 350 μM for wt cells). CEM/Ag-1 cells had a 3-fold reduced capacity to accumulate azidothymidine monophosphate (AZTMP) compared to wt cells whereas similar levels of AZTTP were found in both cell lines. The intracellular half-life of AZTMP was approximately 70 min in both wt and CEM/Ag-1 cells. A 3-fold lower specific activity of cytoplasmic thymidine kinase was observed in CEM/Ag-1 extracts as compared to wt. The reduced thymidine kinase activity was not correlated to a decreased level of thymidine kinase mRNA. Syncytium formation of CEM/Ag-1 cells infected with HIV-2 as well as HIV-1 antigen production was inhibited at the same concentrations of AZT (approx. 0.01 μM) as were HIV-1 and HIV-2 infected wt cells. Thus, minor decreases in cellular thymidine kinase levels may markedly affect the cytoxicity of AZT but have no major effect on the antiviral activity of AZT. Our results strongly suggest that AZTMP is responsible for a major part of the growth inhibitor effects, while AZTTP mainly mediates the antiviral activity of AZT.

Key words: antiviral chemotherapy; cytotoxicity; deoxynucleoside kinases; metabolism; nucleoside analogs

A number of nucleoside analogs inhibit the *in vitro* replication of HIV¶, the causative agent of AIDS [1–3], and AZT has shown definite clinical benefit by reducing morbidity and mortality in AIDS patients [4–7]. The mode of action of AZT is dependent on its phosphorylation by cellular enzymes, i.e. thymidine kinase, thymidylate kinase and nucleoside diphosphate kinase, to yield the corresponding 5′-triphosphate which can compete as substrate with dTTP for the retroviral reverse transcriptase enzyme [8]. Incorporation of analogs with modified 3′-positions leads to termination of the growing viral DNA-chain and antiretroviral selectivity is based on the fact that cellular DNA polymerases bind to these analogs with much lower affinity than viral reverse transcriptase [8–10].

A number of deleterious side effects of AZT have been observed, including anemia and bone marrow suppression [6, 11]. In addition, viruses resistant to AZT have been isolated from patients after treatment for 6 months or longer [12, 13]. Comparative nucleotide sequence analysis of the reverse transcriptase-coding region from sensitive and resistant virus isolates revealed several amino acid substitutions common to resistant virus strains [14].

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¶ Abbreviations: HGPRT, hypoxanthine-guanine phosphoribosyl transferase; AZT, 3′-azidothymidine; AZTMP, 3′-azidothymidine-5′-monophosphate; AZTDP, 3′-azidothymidine-5′-diphosphate; AZTTP, 3′-azidothymidine-5′-triphosphate; dThd, thymidine; dTMP, dThd-5′-monophosphate; dTDP, dThd-5′-diphosphate; dTTP, dThd-5′-triphosphate; TK, thymidine kinase; BrdUrd, 5-bromo-2′-deoxyuridine; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; $CC_{ID_{50}}$, cell culture infective dose-50; Tg, thioguanine; wt, wild type; SSC, standard saline citrate.

In the present investigation we try to identify a correlation between the levels of AZTMP and AZTTP and the cytotoxicity and antiviral activities of AZT. To that end we use a variant of a human T-lymphoblast cell line (CEM/Ag-1) that was found to be partially resistant to the growth inhibiting effect of AZT *in vitro*. Thymidine kinase activity, intracellular azidothymidine metabolite levels, the half-life of azidothymidine nucleotides, AZT transport and the effect of AZT on HIV-1 and HIV-2 replication were measured in these cells. The levels of thymidine kinase mRNA in the wild type cells and in CEM/Ag-1 cells were also determined. In addition, we investigated the effects of AZT on cell growth and HIV replication in a CEM mutant lacking cytoplasmic thymidine kinase activity.

MATERIALS AND METHODS

Chemicals. 3′-Azido-3′-deoxythimidine and thymidine were obtained from the Sigma Chemical Company (St Louis, MO, U.S.A.). Tritiated thymidine (methyl-^{3}H, 25 Ci/mmol) and 3′-azido-3′-deoxythimidine (methyl-^{3}H, 18 Ci/mmol) were from The Radiochemical Center, Amersham, U.K. and Moravek Biochemical Inc., Brea, CA, U.S.A., respectively.

Cell-culture. WT CCRF-CEM cells are well characterized malignant human T-lymphoblastoid cells and the two HGPRT-deficient mutants CEM/Tg-20 and CEM/Ag-1 were both selected for 6-Tg resistance. However these two cell lines were selected at different times and from different passages of parent CEM cells: CEM/Ag-1 was isolated and provided by M. Herschfield, Duke University, Durham, NC, U.S.A., while CEM/Tg-20 was selected in B. Ullmans laboratory relatively recently [15, 16]. The TK-deficient CEM cell line was selected by virtue of its resistance to 50 μM 5-BrdUrd from the CEM/Tg-20 cell line. CEM cells were routinely propagated at 37° in Dulbecco's modified Eagles medium (DMEM) with 10% inactivated (56°, 30 min) horse serum or 7% fetal calf serum in a humidified 7% CO_2 atmosphere and routinely checked for mycoplasma infection.

Growth rate experiments and [methyl-^{3}H]-3′-azidothymidine transport studies. The ability of the CEM cell lines to survive and multiply in cell culture medium containing thymidine and 3′-azido-3′-deoxythymidine was determined after 72 hr incubation as described [15, 16]. The measurements of AZT incorporation into phosphorylated metabolites are affected by transport, metabolism, and efflux. In order to minimize metabolic perturbations to transport measurements, a rapid sampling kinetic assay, which permits the separation of cells from radiolabeled ligand after exposure for time periods as short as 1–2 sec was exploited. Briefly, exponentially growing CEM/wt, CEM/Tg-20 and CEM/Ag-1 cells were harvested by centrifugation and resuspended in growth medium without serum at a density of 5×10^7 cells/mL. To assay the rates of influx of [methyl-^{3}H]-3′-azido-deoxythymidine, 100 μL of transport medium containing 10 μCi/mL 3′-azido-deoxythymidine at 10 μM were layered over 150 μL of a layer of inert oil at 37° as described by Aronow *et al.* [17]. Transport measurements were initiated by rapid addition of an aliquot of the cell suspension to the aqueous phase overlaying the organic layer and terminated by sedimentation in an Eppendorf centrifuge at 10,000 *g* for 25–30 sec. After pelleting the cells, the sample was processed as described previously [17] and counted by liquid scintillation.

Deoxynucleoside kinase assays. TK and deoxycytidine kinase activity in crude extracts of the respective CEM cell lines was measured at 37° in a total volume of 50 μL, containing as substrate 10 μM [methyl-^{3}H]-Thd or [5-^{3}H]-dCyd (specific radioactivity 2 Ci/mmol) or 20 μM [methyl-^{3}H]-AZT (specific radioactivity 2 Ci/mmol) together with 2.5 mM ATP, 2.5 mM $MgCl_2$, 5 mM sodium fluoride, 2 mM dithiothreitol (DTT), 50 mM Tris–HCl (pH 8.0) and 50–100 μg of protein as determined by the Bradford method [18].

dCyd kinase was measured in the presence of 10 μM tetrahydrouridine to inhibit cytidine deaminase activity. Aliquots of the reaction mixture were spotted on a Whatman DE-81 filter paper disk and processed as described [19]. The amount of dCMP, dTMP and AZTMP formed under these conditions was expressed as pmol/min/mg protein and experimental values represent the average value between the three time points, where the assay is considered to be linear with time.

Measurement of intracellular deoxynucleotide concentrations. Exponentially growing CEM cells were incubated at 6–8 × 10^6 cells/mL for different time periods (1–24 hr) with AZT at the indicated concentrations. Cells were harvested by centrifugation at 200 *g* for 10 min at room temperature, followed by two washes in cold PBS. The nucleotides were extracted with 60% cold methanol, and AZTMP was separated and quantitated by HPLC on a C-18 column upon elution with 7.5 mM ammonium phosphate, pH 5.0 plus 7.5% methanol.

When cells were incubated with [methyl-^{3}H]-thymidine or AZT, 2×10^6 cells in 2 mL of medium were incubated for 1–24 hr with different concentrations of AZT (specific radioactivity 6 Ci/mmol). Cells were harvested and nucleotides extracted as described above.

Radiolabeled nucleotides were separated by HPLC, using a Whatman Partisil 10-SAX anion exchange column and a Waters Associates absorbance detector, model 440. When mono-, di- and triphosphates were measured the column was eluted with a gradient (mainly as described in Ref. 20) from 10 mM ammonium phosphate, pH 3.8, 7% methanol (buffer A) to 0.5 M ammonium phosphate, pH 3.8, 7% methanol (buffer B). The gradient ran isocratically for 6 min at a flow rate of 0.75 mL/min using buffer A. From 6 to 13 min the flow changed to 1.5 mL/min, while a linear gradient simultaneously changed buffer from 100% buffer A to 100% buffer B. Buffer B ran isocratically from 10 to 30 min.

All deoxynucleotides were detected by their relative absorbances at 254 nm and 280 nm and quantitated by comparison to known standards or, for the labeled extracts, by their specific activities. Radioactivity measurements on the eluent from the HPLC was done with an on-line radioactivity detector; FLO ONE/Beta Series A-200, Radiomatic

Instruments & Chemical Co., with a liquid scintillator cell, size 2.5 mL, using Insta-gel, Packard Instr., as scintillation liquid.

Half-life determination of AZTMP. Asynchronously growing cells in log phase were incubated for 19 hr with 30 μM AZT. The cells were rapidly washed twice in cold saline with rapid centrifugations in between and resuspended in conditioned medium (obtained from cultures grown under identical conditions in the absence of AZT) at the same cell density as before but without drug added. At various time points after drug removal cells were harvested, nucleotides extracted as described above and the AZTMP quantitated by HPLC.

Determination of the distribution of cells in different cell cycle phases. To measure if the mutant cell lines show different distribution between the different cell cycle phases, approx. 2×10^6 cells were fixed in absolute ethanol and analysed by flow cytometry analysis in a Phywe instrument, Germany [21] by S. Skog and B. Tribukait at the Dept of Radiobiology, Karolinska Institute, Sweden.

Assay for antiviral activity of AZT. The inhibitory effect of AZT on HIV-1 and HIV-2 replication in CEM/wt, CEM/Ag-1, CEM/Tg-20 and CEM/BrdUrd was determined as follows. Virus of the HIV-1 (IIIB) strain was obtained from the persistently infected H9 T-lymphocyte cell line [22]. Uninfected cells were seeded in 24-well microplates with various concentrations of AZT and infected with HIV-1 (four different virus multiplicities were used). Control cells were infected without AZT addition. The cultures were incubated for 6 days without medium change and HIV-1 antigen content determined by immunofluorescence of acetone fixed cells [23] as well as measuring the p24 HIV-1 antigen content in the culture supernatants by enzyme-linked immunosorbent assay as described [24].

Giant cell formation due to syncytium formation by HIV-2 (strain ROD) infection of the CEM cell lines was recorded as described previously [25]. Briefly, CEM cells were suspended at 2.5×10^5 cells/mL of culture medium and infected with HIV-2 at 20 and 5 $CCID_{50}$/mL in the case of CEM/wt or 40 and 10 $CCID_{50}$/mL in the case of CEM/Ag-1. Then, 100 μL of the infected cell suspension was added to 200 μL microtiterplate wells containing 100 μL of an appropriate dilution of AZT. After 4–5 days incubation at 37°, the cell cultures were examined for syncytium formation. EC_{50} was determined as the compound concentration required to inhibit syncytium formation by 50%.

Northern blot analysis of total RNA. Total RNA was prepared after lysis in acid guanidinium-isothiocyanate [26]. Separation of RNA on formaldehyde agarose and transfer to nitrocellulose were done according to standard procedures. Filters were hybridized to cDNA for human cytosolic TK (using the SmaI-BamH1 fragment from pTK11 [27]), human deoxycytidine kinase (NcoI-Bam H1 fragment from pdCK3B1A, kindly provided by B. Mitchell, University of North Carolina, Chapel Hill, NC, U.S.A. [28]) and rat β-actin. Probes were labeled using the nick translation kit provided by the supplier. After hybridization at 42° in a solution containing 25 mM KPO_4, pH 7.6, 5 × SSC, 0.1% SDS, filters were washed at room temperature in 2 × SSC, 0.1% SDS for 4 × 15 min and then at 50° in 0.5 × SSC, 0.1% SDS for 2 × 30 min. Filters were exposed to Kodak XAR film with intensifying screens at −70°.

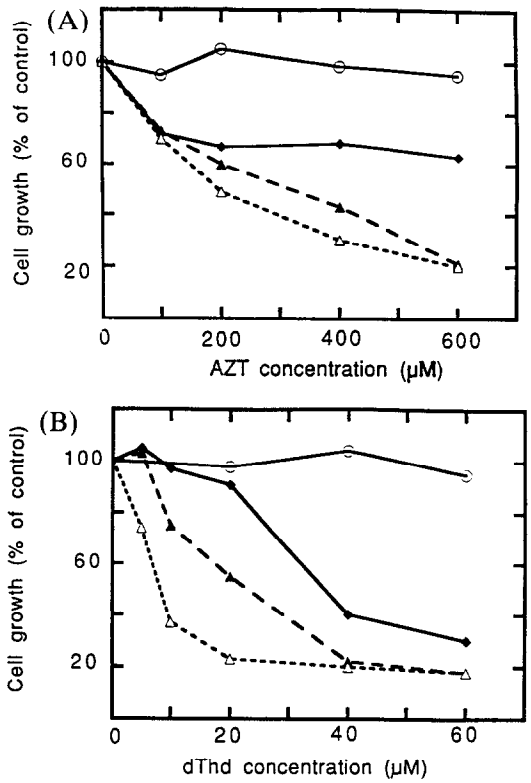

Fig 1. Growth inhibition of CEM/wt, CEM/Tg-20, CEM/BrdUrd and CEM/Ag-1 cells caused by AZT or thymidine. The ability of CEM wild type (△), CEM/Tg-20 (▲), CEM/BrdUrd (○) and CEM/Ag-1 (◆) cells to survive and grow in the presence of increasing concentrations of (A) AZT or (B) dThd was determined. The data shown are those of a single typical experiment that was repeated at least three times with similar results.

RESULTS

Growth rate experiments

The sensitivities of the CCRF-CEM/wt, CEM/Tg-20, CEM/Ag-1, and CEM/BrdUrd cell lines to increasing concentrations of AZT and thymidine are shown in Fig. 1. The effective concentration of AZT that inhibits cell growth by 50% (the EC_{50} value) was 200 and 350 μM for the wt and Tg-20 cells, respectively, while Ag-1 cells exhibited an EC_{50} value of ~2.0 mM. The wt and the Tg-20 mutant cells also showed similar sensitivities to thymidine, 8 and 20 μM, respectively, while the EC_{50} value of CEM/Ag-1 cells to thymidine was 40 μM. CEM/BrdUrd cells were completely refractory to both AZT and Thd at the concentrations tested.

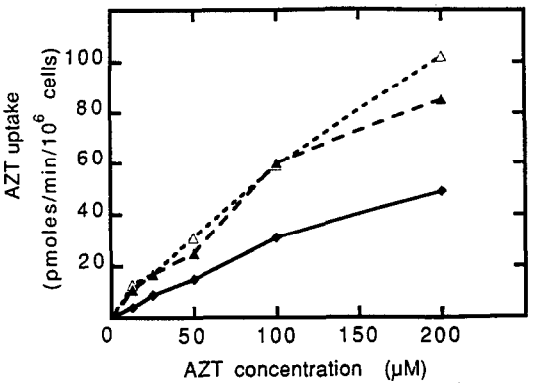

Fig. 2. Transport of AZT into CEM/wt, CEM/Tg-20 and CEM/Ag-1 cells. The uptake of [methyl-^{3}H]-AZT into CEM/wt (△), CEM/Tg-20 (▲) and CEM/Ag-1 cells (◆) was determined for AZT concentrations between 10 and 200 μM, with a rapid sampling kinetic assay, as described in Materials and Methods.

Table 1. Deoxynucleoside kinase activities in crude extracts from CEM/wt, CEM/Tg-20, CEM/BrdUrd and CEM/Ag-1 cells

Cell extract	Activity with dCyd (pmol/min/mg of protein)	dThd	AZT
CEM/wt	195 ± 21	244 ± 97	275 ± 39
CEM/Tg-20	194 ± 51	202 ± 49	170 ± 23
CEM/Ag-1	197 ± 60	94 ± 33	58 ± 4
CEM/BrdUrd	259 ± 18	2.5 ± 0.2	<0.5

Assays were performed with 10 μM dCyd and 10 μM Thd and 20 μM AZT as described in Materials and Methods. Values show the means and the standard deviation (SD) of activity determinations from three independently prepared extracts.

AZT transport studies

In order to assess whether the differential sensitivities of the two HGPRT-deficient cell lines to AZT could be attributed to differences in transport capabilities, their abilities to take up AZT were evaluated by the rapid sampling kinetic assay. As shown in Fig. 2, the CEM/Ag-1 cell line exhibited a significantly lower uptake of AZT than either CEM/wt or CEM/Tg-20 cells.

Deoxynucleoside kinase activity

In order to ascertain whether the differential sensitivities of the various CEM cell lines to thymidine and AZT could be attributed to disparate phosphorylating activities, the activities of TK, as well as deoxycytidine kinase, were evaluated in all strains. Extracts of CEM/Ag-1 cells phosphorylated 10 μM thymidine and 20 μM AZT at rates 40–50% and 20–30%, respectively, of that of CEM/wt and CEM/Tg-20 cell extracts (Table 1). CEM/BrdUrd cells expressed ~1% of the TK activity of the wt cell line with thymidine as substrate. The residual TK activity of CEM/BrdUrd cell extracts could conceivably be attributed to the biochemically and genetically distinct TK activity of mitochondria (TK2) [29].

The apparent K_m value of the cytosolic TK activity from CEM/Ag-1 cells for AZT was ~40 μM, similar to the K_m values obtained with TK from wt cells and for the highly purified TK1 enzyme [29]. Deoxycytidine kinase activity levels were comparable in all four CEM cell lines.

Cell cycle distribution

To determine whether the decreased levels of TK activity in CEM/Ag-1 cells could be attributed to a diminished population of cells in the S phase of the cell cycle, the cell cycle distributions of CEM/Ag-1 and CEM/Tg-20 cells were determined by flow cytometry. No major differences between the two HGPRT-deficient cell lines were observed. The cell cycle distributions were 29.3% in G1 phase, 44.5% in S phase, and 26.3% in G2 + M phases for CEM/Ag-1 cells and 30.6% in G1 phase, 41.5% in S phase, and 27.9% in G2 + M phases for CEM/Tg-20 cells.

Northern blot analysis

To evaluate whether thymidine kinase gene expression was altered in CEM/Ag-1 cells, Northern blot analysis was performed with RNA from CEM/wt, CEM/Tg-20, and CEM/Ag-1 cells (Fig. 3). After normalization by hybridization to the β-actin probe, the three cell lines were shown to express equivalent levels of TK mRNA, as well as deoxycytidine kinase mRNA. Thus, the lower TK activity in CEM/Ag-1 cells must be the result of a post-transcriptional modification.

Measurement of intracellular AZT nucleotide concentrations

AZTMP accumulation was followed with increasing AZT concentration (5–50 μM) after 6 hr of incubation of CEM/wt, CEM/Tg-20 and CEM/Ag-1 cells. The result showed that the parent CEM strain and the CEM/Tg-20 mutant cells accumulated AZTMP approximately 2.5 times better than the Ag-1 mutant at all AZT levels studied (Fig. 4)

To monitor AZT mono-, di- and triphosphate formation as a function of the CEM/Tg-20 and CEM/Ag-1 mutant cells were incubated with [methyl-^{3}H]-AZT (1.6 μM) for 1–24 hr. Throughout the time period studied, the two CEM mutants accumulated equivalent levels of AZT di- and triphosphate, while the AZT monophosphate level was 3–6-fold higher in CEM/Tg-20 cells than in CEM/Ag-1 cells (Table 2). The extent of AZT phosphorylation peaked at 8 hr of incubation both for CEM/Tg-20 and CEM/Ag-1 cells.

The antiviral effects of AZT are observed at submicromolar concentrations. Therefore, the abilities of the two HGPRT-deficient cell lines to phosphorylate AZT at 0.1 μM were evaluated. Table 2 shows that also at this concentration there was no significant difference in the AZTTP levels formed after 8 hr of incubation with CEM/Tg-20 or

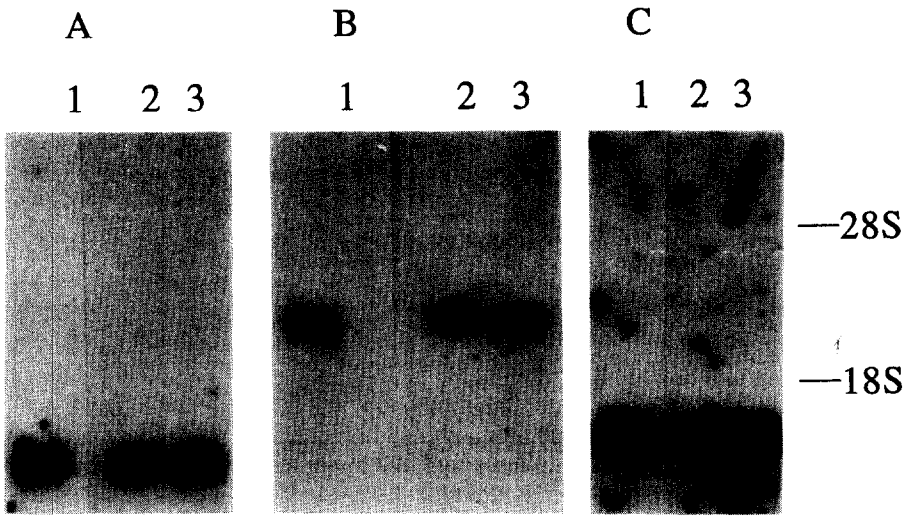

Fig. 3. Northern blot analysis of total RNA from CEM/Tg-20 (1), CEM/Ag-1 (2) and CEM/wt (3) cells. The same filters were hybridized with probes for human thymidine kinase (A), deoxycytidine kinase (B) and rat β-actin (C) as described in Materials and Methods. Autoradiograms were exposed for 4–5 days in A and B and for 16 hr in C.

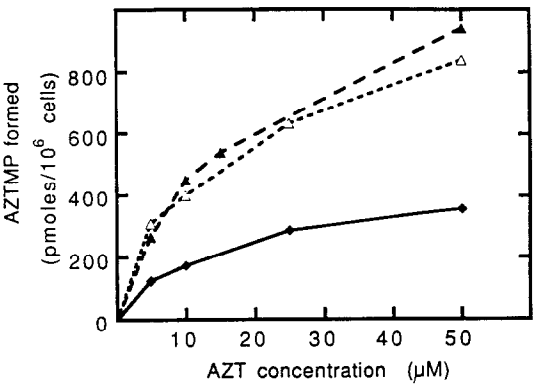

Fig. 4. Intracellular accumulation of AZTMP with increasing concentrations of AZT in CEM/wt, CEM/Tg-20 and CEM/Ag-1 cells. AZTMP levels were HPLC after 6 hr of incubation with 5 to 50 μM AZT and CEM/wt (△), CEM/Tg-20 (▲) and CEM/Ag-1 (◆) cells. The levels represent those of one experiment that was repeated at least twice with similar results.

CEM/Ag-1 cells. However, there was still a 2-fold lower level of AZTMP in CEM/Ag-1 as compared to CEM/Tg-20 cells.

Half-life determinations of AZTMP

To determine if differences in AZTMP accumulation could be due to differences in the breakdown of these compounds, the half-life of AZTMP was examined. After incubation with AZT (30 μM) for 18 hr, the cells were washed and the disappearance of intracellular AZTMP levels was followed at 30 min time intervals for 3 hr. The disappearance of AZTMP exhibited first order kinetics and the $t_{1/2}$ was calculated to be 69 min in both CEM/Tg-20 and CEM/Ag-1 cells (calculations not shown).

Inhibition of HIV-1 and HIV-2 replication

All four CEM cell lines were infected by HIV-1 and HIV-2 and the effects of AZT on virus antigen production measured for HIV-1 by p24 ELISA and immunofluorescence and for HIV-2 by syncytium induction (giant cell formation). Several different HIV-1 multiplicities were tested, and the concentrations of AZT giving 50% inhibition (EC_{50}) of virus antigen production as compared to untreated cells determined (Table 3). The values are shown for the highest virus input where 20–70% of the cells were positive for HIV-1 antigen by immunofluorescence. There were no significant differences in EC_{50} for AZT (concentration of AZT inhibiting HIV-specific antigen expression by 50%) (approx. 0.01 μM) between CEM/wt and CEM/Ag-1 cells, but with CEM/BrdUrd cells there was no inhibition of HIV antigen expression with 10 μM AZT.

When the cells were infected by HIV-2 and scored for giant cell formation (GCF) at day 4–5 post infection, AZT proved an equally potent antiviral agent in CEM/wt and CEM/Ag-1 cells (Table 3). EC_{50} values were 0.01 and 0.006 μM, respectively when 20 $CCID_{50}$ HIV-2 (for CEM/wt) and 40 $CCID_{50}$ HIV-2 (for CEM/Ag-1) were used. At a 5-fold lower

Table 2. AZT metabolites in CEM/Tg-20 and CEM/Ag-1 cells

Cell line	Addition of AZT	Incubation time (hr)	pmoles/10^6 cells AZTMP	AZTDP	AZTTP	ε
CEM/Tg-20	1.6 μM	1	43	0.7	0.3	44.0
		4	81	1.1	0.6	82.7
		8	92	1.4	0.8	94.2
		12	71	1.1	0.6	72.7
		24	60	1.1	0.7	61.8
	0.1 μM	8	9	0.1	0.2	9.4
CEM/Ag-1	1.6 μM	1	13	0.7	0.3	14.0
		4	12	0.5	0.6	13.1
		8	21	0.8	1.1	22.9
		12	15	0.6	0.8	16.4
		24	15	0.6	0.7	16.3
	0.1 μM	8	4	0.2	0.2	4.4

CEM cells were incubated with 1.6 and 0.1 μM AZT for the times indicated, harvested and the intracellular nucleotides extracted with cold 60% methanol. The nucleotides were analysed by HPLC, as described in Materials and Methods. ε represents the sum of all phosphorylated metabolites in pmoles/10^6 cells.

Table 3. Inhibitory effect of AZT against HIV-1 and HIV-2 infection in CEM cell lines

Virus	Assay	EC_{50} for AZT (μM) CEM/wt	CEM/Tg-20	CEM/Ag-1	CEM/BrdUrd
HIV-1	ELISA	0.001	<0.01	<0.01	>10
	IF	0.005	0.01	0.01	>10
HIV-2	GCF	0.01	ND	0.006	>50

The values are from cultures with the highest virus multiplicities giving 25–70% HIV positive cells by immunofluorescence (IF) in the absence of drugs. The lowest AZT concentration tested was 0.01 μM in the case of HIV-1 which gave 60–80% inhibition in the enzyme linked immunoassays (ELISA) for HIV-1 p24 antigen. Giant cell formation (GCF) was estimated under the microscope at day 4–5 post infection. The values are from a single experiment which was repeated twice with similar results.

ND, not determined.

virus input, the EC_{50} values of AZT in both cell lines were again comparable (data not shown).

DISCUSSION

In certain T-cell lines, i.e. H9 and MT-4 cells exposed to very high concentrations of AZT (50–200 μM), decreased phosphorylation of thymidine and decreased levels of dTTP have been reported [8, 30]. This may be attributed to the fact that AZTMP is an alternative substrate–inhibitor of thymidylate kinase. However, decreased concentrations of dTTP were not found [31, 32] with lower concentrations of AZT or in other cell lines, and whether thymidylate kinase inhibition contributes to the bone marrow toxicity induced by AZT is uncertain.

Retroviruses do not encode viral TK activities, or induce increased expression of these enzymes [19]. Therefore a decrease in the activity or level of cellular TK may influence the effectiveness of those antiviral compounds that depend on TK for their phosphorylation.

The CEM/Ag-1 cell line was found to be 6–10-fold resistant to AZT growth inhibition as compared to CEM/Tg-20 and wt CEM cells. CEM/Ag-1 cells were also significantly less sensitive to growth inhibition caused by thymidine, although this difference was not as pronounced. There was no deliberate selection for the Ag-1 phenotype as far as we know and it therefore appears to be a spontaneous variant of CEM cells with lower TK activity than wt CEM cells. A cell line which was selected for its lack of TK activity by resistance to BrdUrd was completely resistant to AZT and Thd growth inhibition.

The relative AZT resistance for cell growth inhibition seen in the CEM/Ag-1 cell line was correlated to a 3-fold lower capacity to accumulate intracellular AZTMP as compared to the CEM/wt and the CEM/Tg-20 cells. Surprisingly, we observed that this reduction in AZTMP was not accompanied by a corresponding reduction in AZT di- and

triphosphate levels, neither at 1.6 μM nor at 0.1 μM concentrations of AZT, which are pharmacologically relevant concentrations.

[Methyl-^{3}H]-3′-azido-3′-deoxythymidine incorporation into phosphorylated metabolites is affected by transport (influx), metabolic and efflux parameters. AZT uptake at different concentrations was compared in the CEM/wt, CEM/Tg-20 and CEM/Ag-1 cell lines; the result indicated that the wt and CEM/Tg-20 cells took up and phosphorylated AZT twice as efficiently as the CEM/Ag-1 mutant cells. This result is most likely explained by the higher phosphorylation rate observed in CEM/wt and CEM/Tg-20 as discussed below.

To clarify whether it was the build up, via the TK, or the break down, via 5′-nucleotidase, or both, that were altered and responsible for the lower AZTMP level in the mutant cells, the half-life of AZTMP as well as TK enzyme activity were determined. AZTMP half-life was similar (70 min) in CEM/Ag-1 and CEM/Tg-20 cells, while TK activity was two–three times lower in CEM/Ag-1 cell extracts both with AZT and dThd as substrates. The BrdUrd-resistant mutant cell line had 100-fold reduced TK activity. The remaining TK activity is most likely due to mitochondrial TK2 in accordance with what was found in other TK-deficient cells [33]. TK2 has a very low capacity to phosphorylate AZT [29], which explains why we did not detect any activity with this substrate in the mutant cell extract. We did not carry out a detailed analysis of AZT metabolism with the BrdUrd-resistant cells, since similar experiments have been done by Balzarini *et al.* [34] with human Raji cells demonstrating a 10,000- and 100-fold lower capacity of TK-deficient cells to accumulate AZTMP and AZTTP, respectively.

The lower TK activity in CEM/Ag-1 cells was not correlated to a difference in mRNA levels, since its expression relative to those of deoxycytidine kinase or β-actin was not decreased. Thus, properties of CEM/Ag-1 cells with regard to the effects of AZT may be due to an altered post-transcriptional regulation of TK leading to a 2–3-fold lower activity of this enzyme with no apparent difference in K_m value. Earlier studies have shown that there were no significant differences in DNA precursor pools between CEM/Ag-1 and CEM/wt cells [35].

The fact remains that the partially AZT-resistant CEM/Ag-1 cell line is markedly less sensitive to the cytotoxic effect of AZT than the AZT-sensitive CEM/wt and CEM/Tg-20 cells, while at the same time the AZTTP pool accumulates to a similar level. This suggests that it is the 5′-monophosphate of AZT that exerts the cytotoxic effect (at least at higher AZT concentrations) and not the 5′-triphosphate derivate of AZT. Also, there was no change in the concentration of AZT required to inhibit HIV-infection in CEM/Ag-1 cells as compared to the other CEM cell lines, which suggests that it is the AZTTP level that is important for the anti-HIV effect.

The lack of a direct correlation between intracellular AZTMP and AZTTP levels in CEM/Tg-20 and CEM/Ag-1 cells, is surprising but earlier studies [36, 37] have also found that increased AZTMP pools are not always associated with increased AZTTP levels. This phenomenon may be due to that thymidylate kinase, which has a K_m for AZTMP of 8 μM [8], is saturated with its substrate already at low cellular monophosphate concentrations. Thus, a 2–3-fold higher level of AZTMP in some cells may have only a marginal effect on the phosphorylation reaction.

One clear cut result here was that the anti-HIV activity of AZT was not significantly different in CEM/Ag-1 cells as compared to CEM/wt and CEM/Tg-20 cells where both HIV-1 and HIV-2 were used in three different assays. However, in the case of the BrdUrd-resistant mutant cells with less than 1% AZT phosphorylating activity, HIV replication was not inhibited at AZT concentrations that were 1000-fold higher than those giving 50% inhibition of virus antigen production in CEM/wt or CEM/Ag-1 cells. These results indicate that TK1 activity is not limiting for the effect of AZT until enzyme activity is drastically reduced.

Recently, Avramis *et al.* [38] described the properties of a T-cell line Jurkat E6-1 that was treated with continuously increasing concentrations of AZT *in vitro*. Several cell isolates capable of growing in 10, 20, 50 and 100 μM AZT were subcloned and shown to have reduced intracellular accumulation of mono-, di- and triphosphate anabolites of AZT. The Jurkat E6-1 cell variants that could grow in medium containing 50 μM AZT had a 15-fold lower specific activity of TK and approx. 10-fold reduced intracellular levels of AZTMP, AZTDP and AZTTP, respectively. Thus, in contrast to what was observed here with CEM/Ag-1 cells, Avramis *et al.* [38] found a direct correlation between TK activity and AZT-nucleotide accumulation. Apparently, the strategy used by these authors led to isolation of cells similar to the CEM/BrdUrd-resistant cells described here.

The growth inhibition determined at very high concentrations of AZT is probably not directly relevant to the bone marrow toxicity observed at much lower concentrations. Cretton *et al.* [39] suggested that it may be the formation of 3′-amino-3′-deoxythymidine that may (partly) explain the *in vivo* toxicity of AZT. Most likely different cell types may be sensitive to different intracellular AZT metabolites, perhaps as a consequence of the expression of altered patterns of anabolic and catabolic enzymes. It is unclear whether a decreased phosphorylation of AZT to AZTMP, as seen in the CEM/Ag-1 cells, can be linked to the formation of other toxic catabolites at lower more relevant physiological AZT concentrations. However, the availability of a human cell line with defined biochemical differences in AZT metabolism may be valuable for the characterization of the biological effects of this antiviral compound.

Earlier studies have shown that there are large variations in the capacity of activated lymphocytes from different individuals to phosphorylate AZT *in vitro* [37]. The results presented here indicate that such variations could be of importance for the cytotoxic effects of AZT. Thus, examination of TK levels could be a useful parameter for the design of individualized AZT treatments with fewer toxic side effects.

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