Expressing engineered thymidylate kinase variants in human cells to improve AZT phosphorylation and human immunodeficiency virus inhibition

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The triphosphorylated form of the nucleoside analogue AZT (AZTTP) acts as a chain terminator during reverse transcription of the human immunodeficiency virus (HIV) genome. The bottleneck in the conversion of AZT to AZTTP is the phosphorylation of AZT monophosphate (AZTMP) by cellular thymidylate kinase. Human thymidylate kinase was engineered to exhibit highly improved activity for AZTMP to AZTDP conversion. It was demonstrated here that genetically modified human cells transiently expressing these enzyme variants show more than 10-fold higher intracellular concentrations of AZTDP and AZTTP. Stable clones expressing these enzymes appear to phosphorylate AZTMP less efficiently, but first experiments indicate they are still more potent in HIV inhibition than the parental cells. It was proposed that the concept of introducing into human cells a catalytically improved human enzyme, rather than an enzyme of viral, bacterial or yeast origin, may serve as a paradigm for ameliorating the metabolic activation of an established drug.

INTRODUCTION

Many drugs such as AZT (3’-azido-3’-deoxythymidine, zidovudine) or the guanosine analogue acyclovir, used against viral infections are precursor molecules (prodrugs) that must be metabolized into their active form by cellular and/or viral enzymes. Their efficiency may be limited by insufficient concentration of the active form due to poor intracellular metabolism. Gene transfer could help to improve both the efficacy and selectivity of these drugs by specifically introducing and expressing a gene in target cells in order to enhance the intracellular activation of the prodrug. We have investigated the potential of this strategy, which is called genetic pharmacomodulation (Caruso & Klatzmann, 1994) or gene-directed enzyme prodrug therapy (Kirn et al., 2002), to improve the antiviral activity of the nucleoside analogue AZT.

AZT is an important antiviral prodrug used in the treatment of human immunodeficiency virus (HIV) infections. The antiviral activity of AZT is due to AZT triphosphate (AZTTP), which acts as a chain terminator during reverse transcription of the viral RNA genome. After uptake of the nucleoside analogue AZT into the cell (Zimmerman et al., 1987), cellular enzymes phosphorylate AZT to its triphosphate form in a stepwise manner (Furman et al., 1986). The first enzyme in this pathway is cellular thymidine kinase (TK), which uses ATP to catalyse the phosphorylation of AZT to its 5’-monophosphate (AZTMP). The second enzyme, thymidylate kinase (TMPK), converts AZTMP to AZT diphosphate (AZTDP), a substrate for nucleoside diphosphate kinase, which is assumed to catalyse the AZTDP to AZTTP phosphorylation. The prodrug AZT is readily converted into AZTTP, which then accumulates in the cell, resulting in low intracellular concentrations of AZTDP and AZTTP (Furman et al., 1986). The bottleneck in AZT activation was shown to be the addition of the
second phosphoryl group catalysed by TMPK (Lavie et al., 1997a).

Our recent structural and biochemical studies of TMPKs from various sources demonstrated that in contrast to yeast and human TMPK, the TMPK from *Escherichia coli* can phosphorylate AZTMP very efficiently (Brundiers et al., 1999). The primary and tertiary structures of the yeast and human TMPKs are quite similar whereas the *E. coli* TMPK exhibits structural differences that appear to be important for its efficient phosphorylation of AZTMP (Lavie et al., 1997a, b, 1998a, b; Ostermann et al., 2000a, b). Kinetic analyses of the enzymes revealed that the catalytic rate constant, $k_{cat}$, for AZTMP phosphorylation is about 35-fold higher with *E. coli* TMPK as compared with yeast TMPK, and about 500-fold higher as compared with human TMPK. A summary of these data is shown in Table 1.

Based on our knowledge of distinctive structural and catalytic properties of eukaryotic and bacterial TMPKs, the human enzyme was engineered in order to improve its AZTMP phosphorylation capacity. Several of the mutant enzymes showed significantly higher AZTMP phosphorylation activities in vitro as compared with the wild-type (wt) enzyme (Brundiers et al., 1999). Two of these mutant human TMPKs were used in our cell culture study presented here: (i) the so-called Large Lid mutant, human TMPK LL, in which the Lid region of the human enzyme (residues 136–148) was replaced by the entire Lid region of the *E. coli* enzyme (residues 142–156). Furthermore, residue Arg-16 was exchanged for Gly (R16G) to mimic the situation in *E. coli* TMPK. (ii) The human TMPK F105Y mutant. Comparison of the kinetic data revealed that the *E. coli* and the yeast enzymes are much faster than human TMPK (Table 1). Structure and sequence analyses have shown that the two faster enzymes possess a tyrosine at position 102 (yeast) or 108 (*E. coli*), respectively. The corresponding residue in human TMPK is Phe-105. Therefore, to improve the catalytic properties of the human TMPK, Phe-105 was replaced by a tyrosine. Indeed, the mutant enzymes showed significantly higher (up to almost 200-fold) AZTMP to AZTDP conversion rates (Brundiers et al., 1999). Notably, the point mutant human TMPK F105Y phosphorylates AZTMP about 20 times faster than does the wt protein and is nearly twice as active on AZTMP as on its natural substrate thymidine monophosphate (TMP). In the present work, we investigated the potential of expressing modified human TMPKs in human cells to enhance anti-HIV activities of AZT.

### METHODS

**Plasmid constructs.** Two mutants of human TMPK were used: F105Y and the so-called Large Lid (LL) mutant (Brundiers et al., 1999). The human TMPK F105Y mutant contains a Phe-to-Tyr substitution at position 105. The LL mutant, human TMPK (R16G; LL) or briefly human TMPK LL, contains the substitution Arg-to-Gly at position 16 and a replacement of its lid region (residues 136–148) was replaced by the entire Lid region (residues 142–156). Furthermore, the coding regions for wt or mutated human TMPKs were isolated from the corresponding pGEX-2T expression vectors by digestion with *NdeI* and BamHI (Brundiers et al., 1999). The fragment was first transferred to the TA cloning vector pCR2.1 (Invitrogen) to obtain the *KpnI* and *NotI* restriction sites necessary for cloning into the mammalian pcDNA expression vector (Promega), in which the recombinant genes are under the control of the enhancer and the immediate-early promoter of the human cytomegalovirus (CMV).

The retroviral integration vector used is a derivative of the Moloney murine leukaemia virus (Mo-MuLV) containing the encapsidation signal $\psi$, the gene encoding the human surface protein Thy-1 (used for flow cytometry detection) and the internal ribosome entry site (IRES) from the encephalomyocarditis virus allowing for the expression of a second recombinant gene from the same transcript. These regions are flanked by the viral Mo-MuLV long terminal repeats (LTRs). The wt or mutant human TMPKs were PCR-amplified to introduce the proper restriction sites and cloned behind the IRES sequence.

**Cell lines and cell culture.** The P4CCR5 cell line, kindly supplied by P. Charneau (Institut Pasteur, Paris, France), is a HeLa-CD4 cell derivative that can be infected with HIV (Charneau et al., 1992). It carries the bacterial lacZ gene under the control of the HIV-1 LTR sequence. P4CCR5 cells also express the CCR5 co-receptor for infection by macrophagic HIV-1 strains and the CXCR4 co-receptor for infection by lymphotropic HIV-1 strains. The expression of $\beta$-galactosidase was used as a marker for HIV infection and was detected 48 h post-infection. The promonocytic leukaemic human cell line U-937 was obtained from the ATCC (Manassas, VA, USA); it expresses the CD4$^+$ receptor but not the Thy-1 surface protein (CD4$^+$ Thy-1$^-$). TE-FLY GA16 (CD4$^+$ Thy-1$^-$), supplied by F.-L. Cosset (Cosset et al., 1995), is a Mo-MuLV based human packaging cell line that produces high titres of retrovirus pseudotyped with gibbon ape leukaemia virus envelope.

The cell lines P4CCR5 and TE-FLY GA16 were grown in DMEM (Dulbecco’s modified Eagle’s medium; GibcoBRL) containing 10% fetal calf serum (FCS), 2 mM L-glutamine (GibcoBRL), 50 μg penicillin ml$^{-1}$, 100 μg neomycin ml$^{-1}$ and 50 μg streptomycin ml$^{-1}$ (GibcoBRL). The U-937 cell line was usually grown in suspension in RPMI 1640 medium (GibcoBRL) with the same additions as above; when co-cultured with the packaging cell line DMEM was used.

**Transfection.** Cells (3×10$^6$) were seeded in a Petri-dish and transfected with a total amount of 20 μg plasmid DNA by using the calcium phosphate method (Sambrook & Russell, 2001). To obtain stable clones we used 1 μg of a plasmid conferring hygromycin

### Table 1. Steady-state phosphorylation kinetics for various thymidylate kinases

<table>
<thead>
<tr>
<th>Organism</th>
<th>TMP</th>
<th>AZTMP</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ [s$^{-1}$]</td>
<td>$K_m$ [μM]</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>15</td>
<td>2.7</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.73</td>
<td>6.3</td>
</tr>
<tr>
<td><em>H. sapiens</em>, wt</td>
<td>1.07</td>
<td>4.2</td>
</tr>
<tr>
<td><em>H. sapiens</em>, mutant Y105F</td>
<td>1.5</td>
<td>22</td>
</tr>
<tr>
<td><em>H. sapiens</em>, mutant LL</td>
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transient resistance and 19 μg of the plasmid containing the recombinant gene. Medium was changed 24 h after transfection, and cells were put under hygromycin selection 48 h after the transfection. After 3 weeks, independent hygromycin-resistant clones were isolated and expanded individually.

Transient transfections of P4CCR5 cells were done following the same procedure, with pCi-human TMPK and pCi-EGFP (encoding enhanced green fluorescent protein) plasmids, using a molecular ratio of 1:1 (3 x 10^13 molecules of each plasmid). Cells were harvested 48 h after transfection. Fluorescence was measured by flow cytometry of appropriate cell aliquots, and cells were then used to analyse thymidine and AZT phosphorylation as described below.

Transient transfections of P4CCR5 cells using the retroviral vector were done with 20 μg plasmid. Cells were harvested 48 h after transfection. The percentage of transfected cells was identified by analysis of Thy-1 expression (see below). Transfection efficiency was >80% in all cases.

Production of stable U-937 clones expressing recombinant human TMPK. Cells of the human packaging cell line TE-FLY GA16 were transfected with the retroviral vector containing wt or one of the mutated human TMPK genes as described above. After 60 h, the medium was removed and 5 ml U-937 cells were added to the culture at a concentration of 1 x 10^6 cells ml^-1 in DMEM culture medium. Protamine sulfate was added at a final concentration of 4 μg ml^-1. After 24 h, U-937 cells from the supernatant were centrifuged (1300 r.p.m. for 5 min at 20°C) and resuspended in DMEM containing protamine sulfate (20 μg ml^-1). One-third to one-half of the cells were again co-cultured with the transfected packaging cells. This procedure was repeated for 10 days. Then the infected cells (CD4+ Thy-1+) were separated from the non-infected (CD4+ Thy-1-) and packaging cells (CD4- Thy-1-) by cell sorting (FACStar Plus; Becton Dickinson). Cells were stained by using a mouse IgG1 anti-human Thy-1 antibody (Genopoint) followed by a secondary goat anti-mouse IgG FITC-conjugated mAb (Caltag) and an anti-human CD4 mAb (CD4-Cy-Chrome). Excitation was done at 488 nm with 5-W argon laser (Coherent Palo Alto) operating at 150 mW. Emission of FITC and Cy-Chrome were measured at 488 nm with 5-W argon laser (Coherent Palo Alto) operating at 150 mW. Emission of FITC and Cy-Chrome were measured at 518 nm and 682 nm, respectively. Cells were sorted at the rate of 3000 events s^-1, with the abort rate being about 10% of the events. CD4+ Thy-1+ expressing cells were collected in 50% FCS and afterwards proliferated in RPMI 1640 culture medium. After several weeks in culture, the cells were repurified, and after further growth the polyclonal population was used for analysis.

Analysis of Thy-1 expression by flow cytometry. Adherent cells were trypsinized, washed with PBS (1 x PBS, 100 mM NaCl, 20 mM Tris/HCl, pH 7.4), and the cell concentration was adjusted to 1 x 10^6 cells ml^-1 for adherent cells or to 3-7 x 10^6 for suspension cells. Cells were incubated for 30 min at 4°C in 100 μl 1 x PBS, BSA (1 g l^-1) and 0.2 g sodium azide l^-1 to which a murine IgG1 anti-human Thy-1 mAb was added in a 100-fold dilution. After washing, cells were incubated with the secondary mAb, FITC-labelled goat anti-mouse IgG-1 (Caltag), for 30 min at 4°C. The number of fluorescent cells and the fluorescence intensity were determined by flow cytometry (FACScanCalibur; Becton Dickinson) using Cellquest software.

Analysis of thymidine and AZT metabolism in cell lines over-expressing human TMPKs. U-937 (5 x 10^6) or P4CCR5 cells (2 x 10^6) were cultivated for 7 h in the presence of 1 μM [methyL-3H]thymidine [5 Ci (185 GBq) mmol^-1; Amersham] or [methyL-3H]AZT [11-7 Ci (432-9 GBq) mmol^-1, Isotopichem]. At the end of the incubation time, the adherent cells were treated with trypsin. Cells were washed three times with 1 x PBS. To extract the nucleotides, the cell pellets were resuspended in 1 ml ice-cold 60% methanol, vortexed and stored at -20°C overnight. After centrifugation for 30 min at 12500 r.p.m. (15000 g) in an Eppendorf centrifuge, the supernatant was lyophilized in a Speed-vac and the dried pellet was resuspended in 500 μl dH2O. After filtration through a 0.22 μm filter (hydrophilic Durapore PVDF low protein binding membrane; Millipore Corporation) the suspension was stored at -80°C.

Different forms of phosphorylated nucleotides were separated by anion-exchange chromatography of the aqueous solution by using a MonoQ HR5/5 column (Pharmacia). The bound nucleotides were eluted with ammonium phosphate buffer (25% ammonium solution, 85% orthophosphoric acid; pH 7.0) by using an improved gradient programme in the concentration range of 0-02-0.5 M phosphate (Guettari et al., 1997; Kremmer et al., 1989). The non-phosphorylated and phosphorylated forms were analysed by counting the radioactivity at the column exit using a Radiomatic Flo-one Beta A-500 counter and were quantified by using the Flo-one/Data software (Radiomatic Flo-one/beta; Packard) (Guettari et al., 1997).

Infection of P4CCR5 cells with HIV-1Lai-791. The HIV-1Lai-791 strain used for infection of P4CCR5 cells was purchased from Diagnostics Pasteur. Its virus titre on P4CCR5 cells was 7.3 x 10^7 TCID50 ml^-1 (tissue culture 50% infectious dose). One day before infection, about 9000 cells per well were seeded into a 96-well microtitre culture plate. On the day of infection, the cells were pre-incubated in triplicate for 5 h with different concentrations of AZT, varying from 10^-3 to 10 μM. Infections were done in a volume of 100 μl (50 μl AZT solution plus 50 μl virus solution) in the presence of 20 μg DEAE-dextran ml^-1. After 24 h, 100 μl new AZT medium was added to the culture. Infections were stopped after 48 h, and β-galactosidase activity of the infected cells was quantified by a chemiluminescent test (Roche Diagnostics). The β-galactosidase activity of cells infected with HIV-1Lai-791 in the absence of AZT was used as a control.

Analysis of AZT toxicity. Non-transfected P4CCR5 cells (5 x 10^6) were cultivated in 6-well plates in the presence of different concentrations of AZT until they were weakly confluent. After 7 days in culture, cells were trypsinized, washed with 1 x PBS and the viable cells were counted by using trypan blue exclusion for detection.

RESULTS

Previous steady-state kinetic data obtained with purified human TMPKs demonstrated that the two mutant enzymes TMPK F105Y and TMPK LL exhibit significantly improved AZTMP phosphorylation activities in vitro (Brundiers et al., 1999). It is important to note that the mutations raised the rate of AZTMP phosphorylation up to almost 200-fold whereas the Km values were only slightly modified. Therefore, the ratio of catalytic efficiency (as given by the value of kcat/Km) for AZTMP versus TDP was elevated drastically from 0.01 in the wt enzyme to 3-5 in the LL mutant (Table 1). The aim of our present work was to determine whether the modified TMPKs are functional and display enhanced catalytic properties when expressed in human cells.

To analyse the engineered TMPKs in human cells we used two cell lines (U-937 and P4CCR5) and two different expression systems: (i) a non-integrating vector containing a CMV promoter leading to constitutive expression of the recombinant gene, and (ii) a retroviral vector harbouring a
double expression cassette resulting in the constitutive expression of a marker gene (encoding the Thy-1 surface protein) and, via an IRES sequence, of a second gene which in our case is human TMPK. Data shown in the figures are representative of two independent experiments. In the first step, we tested the functionality of the mutant enzymes by transient transfection of the corresponding plasmids into a human cell line.

**Functionality of wt and mutant human TMPKs in P4CCR5 cells after transient transfection**

**Transient expression of human TMPKs via the retroviral LTR promoter.** To study the behaviour of the TMPKs in a human cell line, we constructed a retroviral vector that allows both transient transfection of human cells via transfection of the vector containing the retroviral LTR promoter, and, furthermore, infection of human cells in order to introduce the recombinant human TMPK gene stably into the host genome (see below).

The vector we constructed is based on the Mo-MuLV retrovirus. Expression of the recombinant gene was driven by the LTR promoter. The different human TMPK genes were co-expressed with a marker gene encoding the human cell surface protein Thy-1 that can easily be detected on the surface of transfected cells by flow cytometry (see Methods). Moreover, this marker protein would not cause an immune reaction in the human host, an important aspect to consider in potential therapeutic applications. Transfection efficiencies were around 80% in all cases.

To analyse whether the recombinant human TMPK proteins were functional when expressed from this cassette, P4CCR5 cells were transiently transfected, and AZT and thymidine phosphorylation profiles were determined 7 h post-transfection. Fig. 1(a) shows typical elution profiles obtained after FPLC anion-exchange chromatography of the radioactive nucleosides and nucleotides isolated from cells grown in the presence of [³H]AZT. Levels of thymidine triphosphate (TTP) were only slightly increased in cells expressing one of the recombinant human TMPKs indicating that phosphorylation of the physiological substrate is insignificantly affected (Fig. 1b). AZT phosphorylation, however, improved drastically when one of the mutant human TMPK enzymes was expressed (Fig. 1c). The AZTMP proportion dropped from 90% in the parental cells to 36% or 17% in the presence of human TMPK F105Y or human TMPK LL, respectively. Upon expression of the wt enzyme, AZT phosphorylation also increased, though to a much lesser extent, and the AZTMP level (84%) remained almost as high as in the parental cells (90%).

**Transient expression of human TMPKs via the CMV promoter.** The human cell line P4CCR5 (Charneau et al., 1992) was co-transfected with a plasmid encoding one of the human TMPKs and with a plasmid encoding EGFP, allowing us to determine the transfection efficiency by flow cytometry. Transfection efficiency was around 35% for all human TMPK-encoding vectors used. Two days after transfection, cells were grown for 7 h in the presence of [³H]thymidine or [³H]AZT and the radiolabelled

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**Fig. 1.** Transient transfection of P4CCR5 with a retroviral vector. Determination of the nucleosides and nucleotides was performed as in Fig. 1(a) that shows typical elution profiles obtained by FPLC anion-exchange chromatography of total nucleotides extracted from cells that were incubated with [methy]-³H]AZT. Intracellular thymidine (b) and AZT (c) phosphorylation was measured in the human cell line P4CCR5 transiently transfected with a retroviral vector expressing wt or mutant human TMPK as indicated.
nucleosides and nucleotides were extracted and separated by FPLC anion-exchange chromatography.

Cells transfected with wt or mutant human TMPK plasmids showed slightly increased thymidine phosphorylation activities, as indicated by the increased percentage of the phosphorylated species (TMP; thymidine diphosphate, TDP and TTP) in Fig. 2(a). The phosphorylation pattern of the parental cells in Fig. 2(a) is somewhat different from that of Fig. 1(b). This might be due to the fact that endogenous TMPK is a cell cycle-regulated enzyme that is highly expressed only during the S-phase (Huang et al., 1994). Since the cells are not synchronized they might have different phosphorylation patterns.

In the parental cells, most of the [3H]AZT was present in its monophosphorylated form (> 93 %), and AZTDP plus AZTTP represented only about 4 % of the total intracellular AZT (Fig. 2b). Overexpression of the human TMPK wt protein led to a significant reduction of the AZTMP content to 61 %. However, AZTMP was still the predominant phosphorylated species, while AZTDP and AZDTP were present at 17 and 21 %, respectively. A further increase in the levels of AZTDP and AZTTP was accomplished when the mutated human TMPKs were expressed. The relative amount of AZTMP decreased significantly to 37 and 35 % for human TMPK F105Y and human TMPK LL, while the AZTTP levels reached 33 and 36 %, respectively. These results demonstrate that the modified human TMPKs are able to phosphorylate AZTMP in a cellular system and that a much higher level of the antivirally active AZTTP concentration is achieved in these cells.

**Analysis of TMP and AZTMP phosphorylation in stable clones expressing wt or mutant human TMPKs**

The central feature of a future enzyme/prodrug-based antiviral therapy is the delivery of a gene encoding the drug-activating enzyme into human cell lines and permanent integration into the host genome. To obtain more information on the AZTMP phosphorylation efficiency of the recombinant human TMPKs we decided to stably introduce the corresponding genes into the human cell lines U-937 and P4CCR5 in order to determine whether the type of cell and the vector influence the phosphorylation patterns.

**Thymidine and AZT phosphorylation in the human cell line U-937 expressing recombinant human TMPK.**

Retroviral constructs expressing the wt human TMPK or the F105Y mutant were used for transfection of a human packaging cell line, which in turn was co-cultured with U-937 cells. U-937 is a promonocytic leukaemic human cell line that is more similar to the cells of the immune system that are naturally infected with HIV than P4CCR5. Infection of U-937 cells with the viral vector and further purification of the infected cells by cell sorting based on Thy-1 and CD4 expression led to the isolation of a polyclonal population of cells expressing either the wt or the TMPK F105Y mutant protein. Purified cells were proliferated for several weeks and then repurified, again using Thy-1 as a selection marker.

Expression of wt or mutant TMPK did not change the thymidine phosphorylation profile (data not shown). Cells expressing wt TMPK had phosphorylation levels of AZT similar to those of the parental cells. Expression of TMPK F105Y induced a slight but significant increase of the proportions of AZTDP and AZTTP. The relative amounts of AZTDP and AZTTP were lower than in P4CCR5 cells transiently transfected with the same retroviral vector (Fig. 3a).

**Thymidine and AZT phosphorylation in stable human P4CCR5 clones expressing recombinant human TMPKs.**

The P4CCR5 cell line was used for co-transfection with one of the pCi-human TMPK plasmids and a plasmid encoding hygromycin resistance. The pCi vector was chosen in order to transfect the adherent cells with high efficiency whereas infection efficiencies were very low. After antibiotic selection, individual clones were isolated and expanded. Only the plasmids expressing the mutant recombinant enzymes were used for transfection since
they showed high phosphorylation activities in both transient transfections (Figs 1 and 2) and the stable U-937 clones (Fig. 3a). Expression of the two recombinant mutated human TMPKs was verified by detection of their specific mRNAs. About 10% of the hygromycin-resistant clones also expressed the transgene encoding human TMPK LL or F105Y (data not shown). Expression of the transgene did not significantly modify thymidine phosphorylation (data not shown). However, our data clearly show that the percentage of phosphorylated AZT derivatives is reduced (Fig. 3b) as compared with the cells analysed after transient transfection (Fig. 2). Nevertheless, notably with the TMPK F105Y mutant, we still observe a fivefold increased level of AZTDP and AZTTP as compared with the parental P4CCR5 cells. In cells expressing human TMPK LL, the percentages of AZT and its phosphorylated derivatives were, unexpectedly, similar to those observed in the parental cells. Further quantitative measurements of intracellular enzyme activities and/or mRNA levels will have to be performed to determine the molecular basis for these observations.

### Antiviral activity of AZT in a human cell line expressing a mutant human TMPK

To analyse HIV infection of cells that express recombinant human TMPK we used the P4CCR5 cell line. It harbours the bacterial lacZ gene under the control of the HIV-1 LTR and is permissive to HIV infection. The level of infection can easily be followed by determination of the β-galactosidase activity of the culture.

**Cytotoxic effect of AZT.** Since AZTTP can also serve as substrate for cellular polymerases, we first determined whether higher levels of intracellular AZTTP would result in increased AZT toxicity. The cytotoxic effect of AZT, which may also be due to its metabolite AZTMP, was analysed with parental cells and clones stably expressing one of the mutated human TMPKs. Determination of LD50 yielded similar values for the parental P4CCR5 cells and the clones expressing the mutated human TMPKs F105Y and LL (between 20 and 30 μM AZT), indicating no significant modification of AZT toxicity by expression of mutant TMPKs.

**HIV infections of stable P4CCR5 clones.** To see whether the enhanced levels of intracellular AZTTP could be correlated with an improved antiviral activity of AZT, stable clones of P4CCR5 expressing recombinant human TMPK were infected with different dilutions of HIV-1 Lai-791 virus in the presence of increasing concentrations of AZT. Two days after the infection, the β-galactosidase activities of the cultures were measured. Comparison of the AZT concentrations that inhibit virus replication by 50% (IC50), with control cells infected in the absence of AZT, shows that AZT is five to seven times more efficient in preventing HIV replication in stable clones expressing either of the two mutant human TMPKs. Whereas IC50 values for AZT lie between 0.15 and 0.22 μM in the

**Fig. 3.** Stable clones expressing TMPK variants. AZT phosphorylation in stable clones expressing human wt or TMPK mutant enzymes as indicated, in U-937 cells (a) and P4CCR5 parental cells (b).

**Fig. 4.** Effect of AZT on HIV-1 Lai-791 replication in stable clones of P4CCR5 expressing one of the mutant human TMPKs. Percentages of β-galactosidase activities relative to activities of a control infected in the absence of AZT. The IC50 values describe the AZT concentration that inhibits 50% of the infection as compared with the control infected without addition of AZT.
parental cells these values are between 0.03 and 0.04 \( \mu \text{M} \) AZT in cells expressing the TMPK mutants (Fig. 4).

In summary, our results demonstrate that introduction of a modified human TMPK into human cells increases intracellular AZTTP levels such that significantly improved antiviral efficacy of the anti-HIV drug AZT can be observed.

**DISCUSSION**

The aim of our studies was to assess the potential of genetic pharmacomodulation by using a rationally designed, catalytically improved human enzyme and the antiviral produg AZT as a paradigm. We thus analysed whether the expression of modified human TMPKs in human cells can enhance AZTMP phosphorylation in order to overcome the bottleneck in AZT activation (Lavie et al., 1997a) and, as a consequence, significantly raise the level of the antivirally active drug AZTTP, which competes with TTP as a substrate for HIV reverse transcriptase, and inhibit HIV infection in cell culture. Our results clearly demonstrate that AZTMP phosphorylation is significantly improved in two different human cell lines that transiently express recombinant human TMPKs. The elevated intracellular TMPK activity parallels the increased AZTMP phosphorylation activities of the bacterially produced, purified mutant enzymes as observed in *in vitro* enzyme assays (Brundiers et al., 1999). We have used two different transient expression systems that lead to similar levels of high AZTMP phosphorylation capacity. However, with either expression system the situation changes when stable clones are produced. AZTMP phosphorylation is significantly reduced in stable clones of P4CCR5 and U-937 using the pCi derivatives or the recombinant viruses. Similarly, the introduction into HuT 78 or U-937 cells of the Herpes simplex virus thymidine kinase (HSV-1 TK), which can also use AZTMP as a substrate, led to only a slight increase of AZTMP phosphorylation (Guettari et al., 1997). Nevertheless, in either case, pronounced inhibition of HIV replication was achieved.

At present, we do not know at the molecular level the reasons for the less than optimal properties of intracellularly expressed human TMPK mutant enzymes that proved to be highly efficient in AZTMP to AZTDP conversion *in vitro*. Since human TMPK is a regulated enzyme that normally is only expressed during the S-phase of the cell cycle (Huang et al., 1994), continuous overexpression of human TMPK over a long period of time might lead to changes in cellular metabolism. Stable expression of the recombinant gene might therefore select for clones expressing human TMPK at lower levels that do not interfere strongly with the nucleotide metabolism and its regulation. It has been suggested that introduction and overexpression of a modified human TMPK in human cells cannot lead to improved AZTMP phosphorylation since AZTTP and TTP are also allosteric inhibitors of the cytosolic thymidine kinase (TK) at physiological concentrations (Balzarini et al., 1998). Thus, increasing the intracellular AZTTP and TTP levels might have a suppressive feedback effect on the conversion of AZT to AZTMP and, as a consequence, on the synthesis of the antivirally active AZTTP. Yet our results of low AZT levels and high levels of the phosphorylated AZT form in the transient transfections, as well as previous results (Guettari et al., 1997), argue against this scenario. Furthermore, because of similar \( k_m \) values of the two competing substrates TMP and AZTMP, their discrimination by TMPK is determined by the ratio of the rates \( (v_{\text{TMP}} \text{ versus } v_{\text{AZTMP}}) \) at which they are phosphorylated: \( v_{\text{TMP}} / v_{\text{AZTMP}} = (k_{\text{cat}} / k_m)_{\text{TMP}} / (k_{\text{cat}} / k_m)_{\text{AZTMP}} \). Then, at comparable substrate concentrations, the determining factor is the catalytic rate constant, \( k_{\text{cat}} \), which in the case of our best mutant enzyme is higher for AZTTP than for TMP.

We have shown that even a small rise of the intracellular AZT level can result in a significantly increased inhibitory effect on HIV replication. This is expected considering the mechanism of HIV reverse transcriptase inhibition which implies a steep linear dependence on AZTTP concentration (Goody et al., 1991). Alternative interpretations of the increased inhibition of HIV replication may also apply in this system, notably reduced ATP-mediated AZT excision and pyrophosphorolysis (for a recent review of this issue, see Goldschmidt & Marquet, 2004).

Our work also suggests that it could be advantageous to integrate an inducible gene expression system (Rossi & Blau, 1998) that allows for controlled production of the recombinant enzyme. Then, expression of human TMPK would only be switched on when it is actually needed, i.e during HIV infection, but not during the phase of *in vitro* manipulation of the cells to be genetically modified. Upon HIV infection, cells harbouring and overexpressing the recombinant huTMPK gene should be protected against the virus due to their higher intracellular level of inhibitory AZTTP. These cells, being resistant to HIV infection, will have a growth advantage over cells that do not express the recombinant gene and will repopulate the reservoir of cells destroyed by the virus. In patients, ultimately, enhanced AZTMP phosphorylation efficacy should help to suppress virus replication drastically or at least prolong the interval of emergence of drug-resistant viruses. Furthermore, expressing modified human enzymes instead of enzymes of viral, bacterial or yeast origin might be advantageous since an immune response to the introduced human protein appears less likely. Though at the present time the application of gene therapy to the treatment of AIDS may not necessarily be the most convincing example of disease management by gene/enzyme transfer, we believe that our studies on catalytically optimized human thymidylate kinase variants pave the way to the rational design of modified human enzymes for the activation of other compounds applied in chemotherapy. In this line, the replacement of the immunogenic HSV1-TK enzyme, which is widely used in suicide gene therapy of certain cancers,
by an engineered human nucleoside kinase capable of phosphorylating the prodrug ganciclovir selectively and more efficiently would be of prime importance (Bonini et al., 1997; Cohen et al., 1999).

ACKNOWLEDGEMENTS

We would like to thank Karin Vogel-Bachmayr for help with plasmid constructions and Edith Brisson for cell culture work and help with HIV infections. This work was supported by the Max-Planck-Gesellschaft (B. M. W., R. S. G., M. K.), the Deutsche Forschungsgemeinschaft (R. B., M. K.), and by a DAAD-Procope grant (D. K., M. K.). Laurence Loubière was a fellow of Sidaction.

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