Relaxed enantioselectivity of human mitochondrial thymidine kinase and chemotherapeutic uses of L-nucleoside analogues

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Our discovery that Herpes virus thymidine kinase (TK) and cellular deoxycytidine kinase lack enantioselectivity, being able to phosphorylate both D- and L-enantiomers of the substrate, suggested the use of unnatural L-nucleoside analogues as antiviral drugs (Herpes, hepatitis and immunodeficiency viruses). Several L-nucleoside analogues have displayed a short-term cytotoxicity much lower than their corresponding D-counterpart. Since the delayed cytotoxicity of a drug often depends on its effects on mitochondrial metabolism, we have investigated the degree of enantioselectivity of human mitochondrial thymidine kinase (mt-TK). We demonstrate that mt-TK does not show an absolute enantioselectivity, being able to recognize, although with lower efficiency, the L-enantiomers of thymidine, deoxycytidine and modified deoxyuridines, such as (*E*)-5-(2-bromovinyl)-2′-deoxyuridine and 5-iodo-2′-deoxyuridine. Interestingly, the reported negative co-operativity of mt-TK phosphorylating β-D-2′-deoxythymidine (D-Thd), disappears when the deoxyribose moiety has the inverted configuration, resulting in the preferential phosphorylation of D-Thd even in the presence of high concentrations of the L-enantiomer. This, coupled with the higher K_m for β-L-2′-deoxythymidine (L-Thd), makes mt-TK resistant to high concentrations of L-Thd and L-Thd analogues, minimizing the mitochondria-dependent delayed cytotoxicity that might be caused by the administration of L-nucleoside analogues as antivirals.

INTRODUCTION

Recent studies have surprisingly demonstrated that Herpes virus thymidine kinase (TK), unlike to its mammalian cytosolic counterpart, lacks enantioselectivity with respect to D- and L-β-nucleosides [1–6]. Even more surprising is the lack of enantioselectivity of human deoxycytidine kinase (dCK), which phosphorylates both D- and L-enantiomers of deoxycytidine (dCyd) [7] and of dCyd analogues [8,9]. These observations led to the development of a novel class of anti-viral or anti-cancer nucleoside analogues, characterized by the inverted configuration of the sugar ring [1,5,7,10–15], which can be phosphorylated to the monophosphate by Herpes virus TK or human dCK. When further phosphorylated to the triphosphate by cellular kinases, they can inhibit DNA synthesis by interacting with viral or cellular DNA polymerases.

Apparently, these L-nucleoside analogues have a short-term cytotoxicity lower than that of their corresponding D-nucleosides [5,12], although studies on long-term cytotoxicity are still lacking. Since the delayed cytotoxicity of a drug often depends on its effect on mitochondrial metabolism [16], in this work we have investigated the degree of enantioselectivity of mt-TK towards β-L-2′-deoxythymidine (L-Thd), L-dCyd, (*E*)-2-bromovinyl-2′-L-deoxyuridine (L-BVdU) and 5-iodo-2′-L-deoxyuridine (L-IdU) in order to evaluate whether L-nucleoside analogues may be recognized by mitochondrial TK (mt-TK), thus affecting mitochondrial metabolism and contributing to long-term cytotoxicity.

MATERIALS AND METHODS

Chemicals and reagents

β-D-2′-[^{3}H]deoxythymidine ([^{3}H]-D-Thd) (25 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were obtained from Amersham. DEAE 32 resin and DE-81 paper discs were from Whatman. Hi-Trap SP and Mono-Q columns were from Pharmacia. D-Thd, D-dCyd, (*E*)-5-(2-bromovinyl)-D-2′-deoxyuridine (D-BVdU) and D-IdU were supplied by Sigma.

L-Nucleosides

L-Thd was synthesized as described by Spadari et al. [2]. L-BVdU and L-IdU were synthesized as in [5]. L-dCyd was synthesized as described by Holy [17] and Spadari et al. [2]. [^{3}H]-L-Thd (6 Ci/mmol) was obtained from Moravek Biochemicals (radiochemical purity 99.4 %). The L-Thd preparation did not inhibit [^{3}H]-D-Thd (1.12 μM in the assay) phosphorylation by cytosolic TK up to 1 mM [2], indicating that the maximum possible contamination of the 'L preparation' by the D-enantiomer is lower than 0.1 %. Since L-BVdU is obtained from L-IdU by a two-reaction process, which does not involve the chiral centre of the molecule [5], its enantiomeric purity must be, at least, equal to that of the starting L-IdU. For this last compound, the results of the assay with purified HeLa TK [5] showed the maximum possible contamination of the 'L preparation' by the D-enantiomer to be less than 0.3 %.

Purification of mt-TK from HeLa TK$^-$ cells

The HeLa [cytosolic TK negative (TK$^-$)] cell line used to purify mt-TK strictly depends on *de novo* synthesis for the TTP supply and does not incorporate exogenous [^{3}H]Thd into nuclear DNA, proving that cytosolic TK is completely absent in this cell line. Mitochondria were prepared according to the method used by Bolden et al. [18] to isolate mitochondrial DNA polymerase. Briefly, 20 g of cells was resuspended in 100 ml of 10 mM

Abbreviations used: TK, thymidine kinase; mt-TK, mitochondrial thymidine kinase; dCK, deoxycytidine kinase; D-Thd, β-D-2′-deoxythymidine; L-Thd, β-L-2′-deoxythymidine; D-BVdU, (*E*)-5-(2-bromovinyl)-D-2′-deoxyuridine; D-IdU, 5-iodo-D-2′-deoxyuridine; L-BVdU, (*E*)-5-(2-bromovinyl)-L-2′-deoxyuridine; L-IdU, 5-iodo-L-2′-deoxyuridine; D-dCyd, β-D-2′-deoxycytidine; L-dCyd, β-L-2′-deoxycytidine; HSV-1, Herpes simplex virus type 1; DTT, dithiothreitol.

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Tris/HCl, pH 7.6/1 mM dithiothreitol (DTT)/0.5 mM PMSF. After 10 min on ice, cells were homogenized with a Potter homogenizer and nuclei were precipitated by centrifugation for 10 min at 800 ***g***. The supernatant was collected. Nuclei were resuspended in 100 ml of 50 mM Tris/HCl (pH 7.6), 0.25 M sucrose, 1 mM DTT and 0.5 mM PMSF, and washed in a Potter homogenizer. Nuclei were then centrifuged for 10 min at 700 ***g***. The supernatant combined with the previous one (200 ml) was centrifuged for 10 min at 8000 ***g*** to precipitate mitochondria. Mitochondria were further purified by pelleting three consecutive times, in 50 mM Tris/HCl, pH 7.6/0.25 M sucrose/0.5 mM DTT/1 mM EDTA, for 10 min at 8000 ***g***. The washed mitochondria were resuspended in 20 ml of 50 mM Tris/HCl, pH 8/1 M KCl/0.25 M sucrose/1 mM DTT/0.5 mM PMSF, sonicated three times for 5 s at 50 W and then centrifuged for 1 h at 100000 ***g***. The supernatant was filtered on a DEAE 32 column (3 ml), dialysed against 50 mM Tris/HCl, pH 8/1 mM DTT/0.5 mM PMSF, and then passed through a Hi-Trap SP column (1 ml). The unbound material was immediately loaded on a Mono-Q column (1 ml) at 0.5 ml/min. The enzyme was eluted by a linear gradient between 0 and 0.6 M KCl in the previous buffer. Fractions containing mt-TK were pooled and stored in liquid nitrogen.

TK assays

mt-TK was assayed at 37 °C in 25 μl of 50 mM Tris/HCl (pH 8), 5 mM $MgCl_2$, 5 mM ATP, 2 mM DTT, 10 mM NaF and 4 μM [^{3}H]Thd (25 Ci/mmol) or 4 μM [^{3}H]-L-Thd (6 Ci/mmol). The reaction was stopped by spotting 20 μl of the incubation mixture on a 25-mm DE-81 paper disc. Discs were washed three times in an excess of 1 mM ammonium formate, pH 3.6, in order to remove unconverted nucleoside, and finally twice in ethanol. Discs were then placed in 20 ml scintillation vials. A 1 ml volume of 1 M KCl/0.1 M HCl solution was added and radioactivity was extracted by shaking for 20–30 min. To each vial 4 ml of EcoLume (ICN) was added and the radioactivity was counted in a β-counter. When [γ-^{32}P]ATP was used in mt-TK assays, the enzyme was incubated in the mixture described above containing 0.2 mM [γ-^{32}P]ATP (1000 c.p.m./pmol), 2 mM $MgCl_2$ and 40 μM of the nucleoside to test. After 1 h at 37 °C samples were heated for 5 min at 80 °C and centrifuged at 8800 ***g***. A 20 μl portion of the supernatant was injected for HPLC.

Nucleoside and nucleotide separation by reverse-phase HPLC

The reverse-phase method employing HPLC was used in order to separate nucleosides from nucleotides. A 0.4 cm × 15 cm reverse-phase C_{18} BioSil ODS-5S column was used at room temperature under the following conditions: injection volume, 20 μl; detection, UV 260 nm; eluents, buffer A (20 mM KH_2PO_4, pH 3.6), buffer B (20 mM KH_2PO_4, pH 3.6/60 % methanol). Gradient conditions were as follows: 0–10 min, 0 % buffer B; 10–16 min, 0–100 % buffer B; 16–33 min, 100 % buffer B, at a flow rate of 0.5 ml/min. Thirty-three (fraction volume: 0.5 ml) were collected and counted in a β-counter.

RESULTS

Phosphorylation of D- and L-Thd by purified mt-TK

In order to obtain mt-TK without contamination by cytosolic TK, which is the main Thd phosphorylating activity in human cells, we have purified mt-TK activity from the mitochondrial fraction of TK$^-$ HeLa cells, as described in the Materials and methods section.

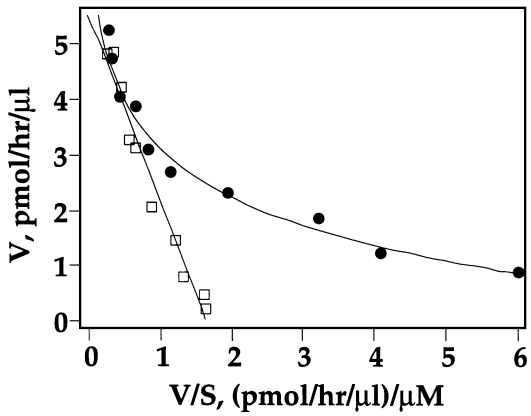

Figure 1 Activity of mt-TK at various D-Thd (●) and L-Thd (□) concentrations

The data are plotted according to the method of Hofstee. Regression analyses were used for calculation of the kinetic parameters. Each point represents the mean of triplicate determinations and the S.D. for each point is lower than 10%.

Table 1 Kinetic parameters of mt-TK for D-Thd and L-Thd

LS, low substrate concentrations (< 1.5 μM); HS, high substrate concentrations (> 1.5 μM). Values ± S.D. from three experiments are given.

	K_m (μM)		V_{max} (pmol·h^{-1}·μl^{-1})		V_{max}/K_m (pmol·h^{-1}·μl^{-1}·μM^{-1})	
	LS	HS	LS	HS	LS	HS
D-Thd	0.37 (±0.07)	7.22 (±0.8)	3.00 (±0.3)	7.14 (±0.4)	8.10	0.98
L-Thd	4.4 (±0.2)	4.4 (±0.2)	5.7 (±0.2)	5.7 (±0.2)	1.29	1.29

Cytosolic TK is strictly enantioselective and does not recognize the L-enantiomer of the natural substrate D-Thd [2]. Cytosolic dCK, which phosphorylates deoxycytidine, deoxyguanosine and deoxyadenosine, but not deoxythymidine [19], shows a lack of enantioselectivity for deoxycytidine and several deoxycytidine analogues, but is unable to recognize several other L-nucleosides, including L-Thd, L-deoxyadenosine, L-deoxyguanosine, L-deoxyuridine, L-BVdU and L-IdU [7].

To study the enantioselectivity of mt-TK we compared the ability of this enzyme to phosphorylate D- and L-Thd by measuring the initial velocity of reaction at various substrate concentrations (Figure 1). The kinetic constants, calculated by non-linear regression analysis, are shown in Table 1. At substrate concentrations lower than 1.5 μM, D-Thd is a much better substrate than L-Thd, as judged by the lower K_m (0.37 compared with 4.4 μM) and higher V_{max}/K_m (8.10 compared with 1.29 pmol·h^{-1}·μl^{-1}·μM^{-1}). At higher substrate concentrations, mt-TK phosphorylates both D- and L-Thd enantiomers with similar efficiency (Figure 1), as demonstrated by comparable K_m values (7.22 μM compared with 4.4 μM), V_{max} (7.14 pmol·h^{-1}·μl^{-1} compared with 5.7 pmol·h^{-1}·μl^{-1}) and V_{max}/K_m values (0.98 compared with 1.29 pmol·h^{-1}·μl^{-1}·μM^{-1}) reported in Table 1. Interestingly, the kinetics with L-Thd was of the Michaelis–Menten type, as indicated by the straight line Hofstee plot shown in Figure 1 (Hill coefficient 0.98), while the kinetics with D-Thd showed the negative cooperativity (Hill coefficient 0.35) already observed by Munch-Petersen et al. [20], which

Table 2 IC_{50} values of mt-TK for D- and L-nucleosides

Each IC_{50} value represents the mean of three experiments in which each point is from duplicate measurement. IC_{50} values were determined at 4 μM [^{3}H]-D-Thd as described in the Materials and methods section.

Compound	IC_{50} (μM)
D-BVdU	1.5
D-IdU	26.0
D-dCyd	172.0
L-Thd	126.0
L-BVdU	70.0
L-IdU	400.0
L-dCyd	662.0

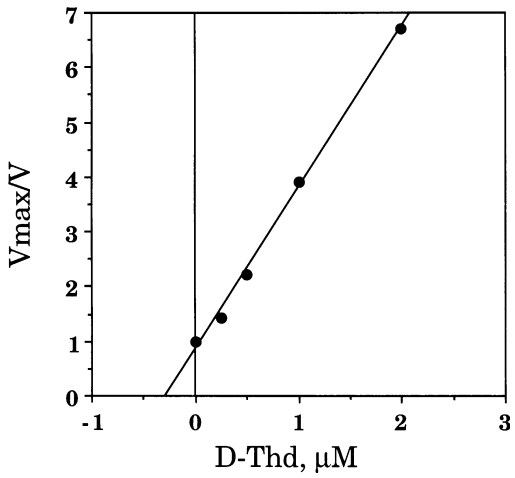

Figure 2 Effect of D-Thd on the phosphorylation of [^{3}H]-L-Thd by mt-TK

The concentration of [^{3}H]-L-Thd present in the assay was 4 μM. Each point represents the mean of triplicate determinations and the S.D. for each point is lower than 10%.

suggests that mt-TK binds successive D-Thd, but not L-Thd, molecules with decreasing affinity.

Substrate competition

The negative co-operative kinetics observed in the phosphorylation of D-Thd and the simple Michaelis–Menten mechanism observed in the phosphorylation of L-Thd suggested studying the competition between D- and L-Thd by inhibition analysis. When L-Thd was used as a competitor of [^{3}H]-D-Thd (4 μM) phosphorylation by mt-TK, we observed a surprisingly high IC_{50} value (126 μM, Table 2). This suggests that the binding of L-Thd to the enzyme occurs without decreasing the affinity of the active site for [^{3}H]-D-Thd. In other words, at high concentrations of L-enantiomer, the binding of L-Thd to the enzyme overcomes the negative co-operativity observed with D-Thd alone. This results in permanent high affinity for D-Thd, with consequent preferential phosphorylation of the D- compared with the L-enantiomer in racemic solutions. These data appear to be confirmed by the fact that, when the enzyme is phosphorylating [^{3}H]-L-Thd, a concentration of 0.3 μM D-Thd (corresponding approximately to its K_m value) is already sufficient to inhibit the enzyme activity by 50% (Figure 2).

Finally, D-dCyd, which is phosphorylated by mt-TK with a K_m of 6 μM (results not shown), inhibits the phosphorylation of [^{3}H]-D-Thd with an IC_{50} value of 172 μM (Table 2), also suggesting that in the presence of other pyrimidine nucleosides, the enzyme active site maintains high affinity for its natural substrate D-Thd.

Effect of L-nucleoside analogues and L-deoxycytidine on the phosphorylation of D-Thd by mt-TK

D-BVdU, whose potency against Herpes simplex virus type 1 (HSV-1) replication in cell culture and *in vivo* is remarkable, shows potential drawbacks, such as affinity for the mt-TK [21], inhibition of thymidylate synthase by its monophosphate [22] and degradation by thymidine phosphorylase [23]. On the other hand, we have found that L-BVdU is 1000-fold less cytotoxic towards HeLa TK$^-$ cells transformed by the HSV-1 TK gene (HeLa TK$^-$/HSV-1 TK$^+$) than its D-enantiomer [5]. This is mainly due to the fact that L-BVdUMP, produced from L-BVdU by HSV-1 TK, has a much lower inhibitory effect than D-BVdUMP on host thymidylate synthase [5]. However, in spite of its very low cytotoxicity, L-BVdU is only two orders of magnitude less potent than its D-enantiomer in inhibiting viral growth, with potency comparable with that of Acyclovir [5].

Because delayed cytotoxicity of a drug often depends on its effect on mitochondrial metabolism, we studied the effect of L-BVdU and of other L-nucleosides, such as L-IdU and L-dCyd, on mt-TK. When different concentrations of L-BVdU, L-IdU and L-dCyd were tested on purified mt-TK we observed that L-BVdU inhibits the phosphorylation of [^{3}H]-D-Thd by mt-TK about 50-fold less than its D-enantiomer (Table 2). Also, L-IdU and L-dCyd show IC_{50} values higher (15-fold and 4-fold respectively) than those observed for the D-enantiomers (Table 2).

To verify whether L-BVdU, the L-analogue with the lowest IC_{50} value, is a substrate like its D-enantiomer [24], or only a non-substrate inhibitor of mt-TK, we incubated mt-TK and [γ-^{32}P]ATP, as phosphate donor, with 40 μM L-BVdU (close to the IC_{50} value), a concentration which should promote the formation of its monophosphate. As a control, D-BVdU, which is known to be a substrate of mt-TK, was phosphorylated under the same assay conditions. Figure 3 shows the HPLC elution profile of the products of the reaction (D- and L-BVdUMP) of mt-TK with D- and L-BVdU. The retention time of the putative L-BVdUMP (Figure 3B) is identical with that of D-BVdUMP (Figure 3A). Therefore, HPLC analysis demonstrated that D- and L-BVdU are phosphorylated with comparable efficiency by mt-TK. These results are consistent with the catalytic behaviour of the enzyme. In fact, D-BVdU and L-BVdU are present in the assay mixture at high concentrations, a condition which, as observed for D- and L-Thd (see Figure 1), could cause the enzyme to show similar K_m and V_{max} values for both enantiomers and therefore comparable efficiency of phosphorylation.

DISCUSSION

This study demonstrates that mt-TK, in contrast with cytosolic TK, shows a somewhat relaxed enantioselectivity, being able to recognize L-nucleosides such as L-Thd, L-dCyd, L-BVdU and L-IdU. Its degree of enantioselectivity is nevertheless higher than that of Herpes virus TK [2,5] and human dCK [7]. In fact, in contrast with Herpes virus TK and human dCK, which have comparable K_m values for the D- and L-enantiomers of the respective substrates, mt-TK has a K_m for L-Thd 12-fold higher than for its D-enantiomer. Furthermore, the IC_{50} values of the nucleoside analogues L-dCyd, L-IdU and L-BVdU for the phosphorylation of D-Thd by mt-TK are 4-, 15- and 50-fold higher than those of their D-counterparts respectively. Our studies have also confirmed

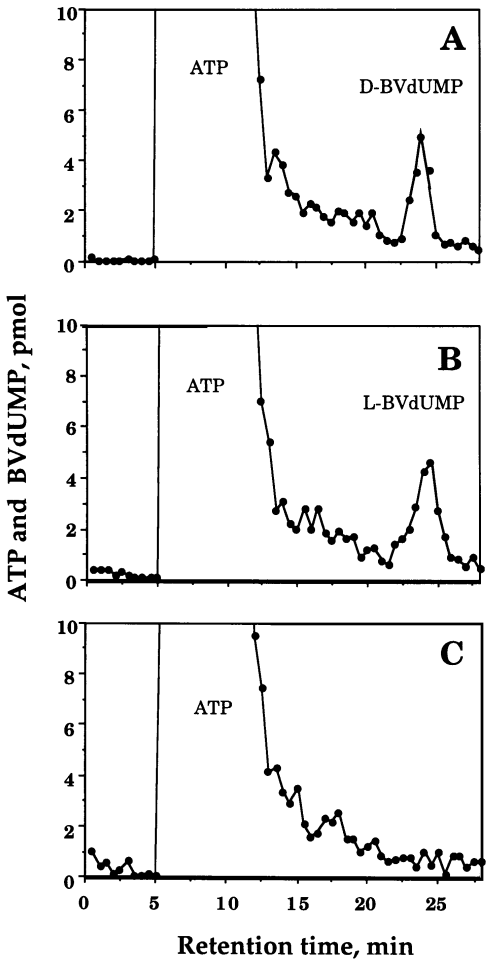

Figure 3 HPLC analysis of the reaction products of mt-TK with [γ-^{32}P]ATP and either D-BVdU (A), L-BVdU (B) or control (C)

Mt-TK reaction and resolution of reaction products were performed as described in the Materials and methods section. In each case a typical elution profile, representative of three, is shown.

the negative co-operative kinetics of the enzyme in the phosphorylation of the natural substrate [20], but interestingly, the negative deviation from ideal behaviour disappears when the deoxyribose moiety has the inverted configuration. This results in preferential phosphorylation of D- compared with the L-nucleosides in a racemic solution.

It is intriguing that mt-TK not only shares with herpetic TKs and human dCK the ability to phosphorylate dCyd [25], but also a limited enantioselectivity. For herpetic TKs and human dCK a considerable degree of genetic homology was found, suggesting a common evolutionary origin [26]. However, because to our knowledge no sequence data are available for mt-TK, the relationships between this enzyme and other TKs are still not clear [27]. Crystallographic studies on the interaction of the L- and D-enantiomers of the natural substrates with the active site of these enzymes would therefore be enlightening, possibly revealing common features in their active sites.

From the chemotherapeutic point of view the preferential phosphorylation of D-Thd, even in the presence of very high concentrations of the L-enantiomer, coupled with the higher K_m for L-Thd, would contribute to minimizing mitochondria-dependent delayed cytotoxicity possibly associated with the administration of L-nucleoside analogues, and would not compromise the chemotherapeutic efficacy of novel antiviral L-nucleoside analogues.

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