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Phosphorylation of the Anti-Hepatitis B Nucleoside Analog 1-(2′-Deoxy-2′-Fluoro-1-β-D-Arabinofuranosyl)-5-Iodouracil (FIAU) by Human Cytosolic and Mitochondrial Thymidine Kinase and Implications for Cytotoxicity

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The capacity of recombinant human cytosolic thymidine kinase (TK1) and bovine mitochondrial thymidine kinase (TK2) to phosphorylate the antiviral analogs 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil (FIAU) and 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-methyluracil (FMAU) has been analyzed. The V_{max}/K_m ratios for FIAU and FMAU with TK2 are about 30% of that for deoxythymidine, while the corresponding values for TK1 are 2 and 5%, respectively. Thus, these two analogs are more efficient substrates for TK2 than for TK1, which may be part of the explanation for the mitochondrial toxicity associated with FIAU during treatment of hepatitis B infection.

2′-Fluorinated arabinosyl pyrimidine nucleosides have shown high antiviral activity against herpesvirus, cytomegalovirus, and hepatitis B virus infections, and cellular toxicity was observed only at high nucleoside concentrations (5, 6, 10, 13, 16, 18). Among them, 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil (FIAU) and one of its in vivo metabolites, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU), have been found to exhibit a high capacity to suppress the replication of hepatitis B virus in treated animals and patients (6, 10, 13, 16). Nevertheless, when FIAU was orally applied to 15 patients with chronic hepatitis B, unanticipated liver toxicity was observed weeks later in 10 patients, of whom seven suffered severe toxicity and five died (reviewed in references 6, 13, and 16).

The clinical symptoms indicated that there was mitochondrial toxicity, i.e., the interference with mitochondrial (mt) DNA synthesis and function, as a result of FIAU treatment. Inhibition of mtDNA synthesis has also been observed during treatment with antiviral dideoxynucleosides such as zidovudine, ddI, and ddC, but in contrast to those analogs which serve as chain terminators for mtDNA synthesis, FIAU triphosphate (TP) can be efficiently incorporated into mtDNA (6, 13, 16). This mechanism relies on efficient incorporation of nucleoside analogs into mtDNA by the mtDNA polymerase, which is known to have low selectivity and high activity with dideoxynucleoside triphosphates and FIAU-TP (14). Incorporation of FIAU-TP then leads to inhibition or dysfunction of mtDNA synthesis, since there is no efficient repair mechanism to remove FIAU monophosphate (13, 16).

A prerequisite for this mechanism is the phosphorylation and transport of nucleoside metabolites into mitochondria. Hepatitis B virus does not code for a nucleoside kinase, but there are two cellular nucleoside kinases capable of catalyzing the first step in the anabolism of FIAU: cytosolic thymidine kinase (TK1) and mitochondrial thymidine kinase (TK2). The latter is expressed at a low but constant level in all cells, while TK1 is found highly expressed only in S-phase cells (reviewed in reference 1).

Cheng et al. (3) have shown that both TK1 and TK2 can phosphorylate FMAU and FIAU, but the enzymes in that study were not purified, and only one high concentration of the substrates was tested. Recently, purification of TK2 to homogeneity from human or bovine sources has been developed (12); expression of human TK1 cDNA (11) in *Escherichia coli* has been achieved as well, and it will be presented here shortly. The availability of pure preparations of TK1 and TK2 enabled us to investigate some of the kinetic properties of FIAU and FMAU with these two enzymes in detail and to discuss their antiviral and toxic effects on the basis of such results.

Chemicals. [γ-^{32}P]ATP was from Radiochemical Centre, Amersham, United Kingdom. FIAU and FMAU were synthesized and provided by J. Fox, Memorial Sloan Kettering Cancer Institute, New York.

Enzyme preparation. A T7 gene 10 fusion protein was constructed by cloning the *Sma*I and *Bam*HI restriction fragment of human TK1 (nucleotides 110 to 1343 [2]) into the pT7-7 vector (17), thereby substituting the N-terminal 16 amino acids of TK1 with 5 amino acids from the T7 gene 10 protein, which gives a 24-kDa fusion protein. This plasmid, designated phTK1, was transfected into *E. coli* BL21(DE3) LysS, and production of the T7 gene 10 fusion protein was induced with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 25°C for 3 h (10).

Cells were resuspended in buffer A (25 mM Tris-HCl [pH 7.0], 1 mM EDTA, 1 mM dithiothreitol, 0.05 M KCl, 20% glycerol, 0.1% Triton X-100) and a protease inhibitor, phenylmethylsulfonyl fluoride (2.5 mM). The suspension was freeze-thawed three times and sonicated; this was followed by centrifugation for 45 min at 100,000 × *g* at 4°C. The crude extract contained approximately 10 mg of total protein per ml and was diluted with an equal volume of buffer A and applied directly to a MemSep SP1010 (Millipore) column attached to a Millipore ConSep unit. The column was eluted with a 0.05 to 0.5 M linear KCl gradient in buffer A without EDTA and the protease inhibitor. A substantial fraction of TK1 activity was found in the flowthrough collection, but nearly 40% of the total TK1

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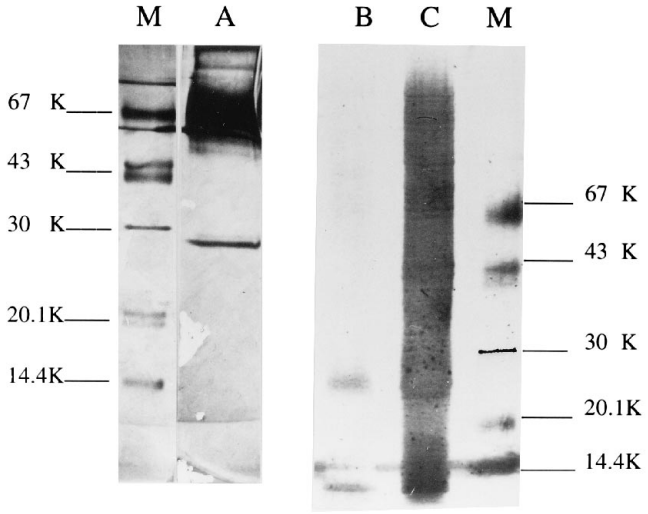

FIG. 1. SDS-gel electrophoresis of TK2 from bovine brain and recombinant human TK1. The 10 to 15% gradient gel was visualized by silver staining. M, molecular weight standards; A, TK2 preparation (with the addition of bovine serum albumin); B, recombinant TK1 preparation; C, crude extract of recombinant TK1.

activity was eluted with 0.2 M KCl. The fraction with the highest TK1 activity contained a polypeptide of 24 kDa as revealed by sodium dodecyl sulfate (SDS)-gel electrophoresis, and this preparation was approximately 90% pure (Fig. 1).

TK2 was purified from bovine brain extracts, as described in reference 15. The overall structure and specificity of bovine TK2 and recombinant TK1 are very similar to those of the human spleen enzymes (12, 15).

Enzyme assays. The nucleoside substrates were tested as phosphate acceptors with 100 μM [γ-^{32}P]ATP as the phosphate donor, 50 mM Tris-HCl (pH 7.6), 0.5 mM $MgCl_2$, 100 mM KCl, and 0.5 mg of bovine serum albumin per ml at 37°C for 10 to 30 min. The products were analyzed by polyethyleneimene-cellulose thin-layer chromatography, the X-ray films were quantified with an LKB Ultroscan XL laser densitometer, and the kinetic constants were determined with the Enzfitter software from Elsevier-Biosoft (7).

Purification of recombinant human TK1 from overproducing *E. coli* cells by ion-exchange chromatography (11) led to a preparation that contained essentially pure TK1 polypeptide (Fig. 1). The specific activity and overall properties of this recombinant enzyme are very similar to those of the earlier-studied enzyme from human spleen or lymphoblast (15).

TK2 was purified from bovine brain extracts by DEAE and hydroxylapatite chromatography; this was followed by affinity chromatography on dTMP-Sepharose as previously described (15). The final preparation contained only one band of 29 kDa (bovine serum albumin was added to stabilize the TK2 enzyme) (Fig. 1). The properties of the bovine and human enzymes are very similar.

A direct phosphorylation assay was used, and the formation of radioactive monophosphate product was monitored by using [γ-^{32}P]ATP as the phosphate donor (7). The difference in the capacities of TK1 and TK2 to use FIAU and FMAU was apparent when the K_m and V_{max} values were determined (Table 1). The K_m values for these two analogs with TK1 were 35- and 5-fold higher than those with TK2, while the relative efficiency (V_{max}/K_m) for each analog was approximately 30% of that for deoxythymidine in the case of TK2 but only 2 and 5%, respectively, in the case of TK1 (Table 1). The discrepancy in absolute specific activities between the enzymes used here and those used in the previous studies (15) is primarily due to the difference in assay conditions.

TABLE 1. Kinetic parameters for TK1 and TK2 with deoxythymidine, FIAU, and FMAU[a]

Enzyme	Substrate[b]	K_m (μM)	V_{max} (pmol min^{-1} mg^{-1})	V_{max}/K_m[c]
TK1	Thd	2.1 ± 0.4	2,100 ± 300	670
	FMAU	33 ± 1.5	1,000 ± 300	31 (4.6)
	FIAU	140 ± 38	1,600 ± 200	11 (1.7)
TK2	Thd	1 ± 0.12	150 ± 40	150
	FMAU	7 ± 0.2	265 ± 30	38 (25.3)
	FIAU	4 ± 0.6	200 ± 40	50 (33)

[a] Data were obtained by using the phosphotransfer assay with 100 μM of [γ-^{32}P]ATP–0.5 μM $MgCl_2$.
[b] Thd, deoxythymidine.
[c] Values in parentheses are percentages of the value obtained with Thd for the given enzyme.

The tissue selectivity of the toxic side effects of deoxynucleoside analogs may come from the different capacities of different cells and tissues to phosphorylate the analogs. There is also a large variability in the expression of the cellular deoxynucleoside kinases, and as stated earlier, TK1 is found only in proliferating cells while TK2 is expressed in all cells but at a level 10- to 100-fold lower than the maximal TK1 level (1).

FIAU and FMAU are substrates for TK1 and even better substrates for TK2, but since the V_{max} of TK1 is higher than that of TK2, it is likely that the in vivo anabolism of these analogs occurs through both enzymes. These results agree with those of Cheng et al. (3) and other earlier work showing that TK2 has a high capacity to phosphorylate arabinosyl thymidine as well as 5-halogen- and 5-heteroaryl-substituted deoxypyrimidine analogs (8). However, in a recent study by Cui et al. (4), no formation of phosphorylated metabolites was detected when purified mitochondria were incubated with radioactive FIAU. We do not know why TK2 was not capable of activating FIAU in that experiment, but we have observed earlier that although mitochondrial extracts show a higher TK2 specific activity than cytosolic extracts, 80% of the total activity occurs in the cytosol (12). Therefore, it is possible that isolated mitochondria are at least partially deficient in TK2 and have a low capacity to activate deoxynucleoside analogs compared with the situation for intact cells.

It is likely that there is an association between the relatively efficient phosphorylation of FIAU by TK2 and the mitochondrial toxicity observed with this nucleoside. The broad specificity of the mitochondrial thymidine kinase and mtDNA polymerase (9, 14) is of significant concern in the design of new antiviral nucleoside analogs. Clearly, there are other important processes and enzymes responsible for the incorporation and inhibitory effects of FIAU-TP or FMAU-TP, such as the catabolism of the nucleotides, their rate of incorporation by the mtDNA polymerase, as well as the eventual excision and repair of the altered mtDNA. We have focused here on one aspect of this process, and even though more basic biochemical information is necessary for future reliable drug design, it seems advisable to screen candidate chemotherapeutic analogs as substrates for key enzymes in mtDNA metabolism in order to identify at the preclinical state potential toxic inhibitors of this process.

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