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Expression of deoxynucleoside kinases and 5′-nucleotidases in mouse tissues: Implications for mitochondrial toxicity

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ABSTRACT

Anti-HIV nucleoside therapy can result in mitochondrial toxicity affecting muscles, peripheral nerves, pancreas and adipose tissue. The cytosolic deoxycytidine kinase (dCK; EC 2.7.1.74) and thymidine kinase (TK1; EC 2.7.1.21), the mitochondrial thymidine kinase (TK2) and deoxyguanosine kinase (dGK; EC 2.7.1.113) as well as 5′-deoxynucleotidases (5′-dNT; EC 3.1.3.5) are enzymes that control rate-limiting steps in formation of intracellular and intra-mitochondrial nucleotides. The mRNA levels and activities of these enzymes were determined in mouse tissues, using real-time PCR and selective enzyme assays. The expression of mRNA for all these enzymes and the mitochondrial deoxynucleotide carrier was detected in all tissues with a 5–10-fold variation. TK1 activities were only clearly detected in spleen and testis, while TK2, dGK and dCK activities were found in all tissues. dGK activities were higher than any other dNK in all tissues, except spleen and testis. In skeletal muscle dGK activity was 5-fold lower, TK2 and dCK levels were 10-fold lower as compared with other tissues. The variation in 5′-dNT activities was about eight-fold with the highest levels in brain and lowest in brown fat. Thus, the salvage of deoxynucleosides in muscles is 5–10-fold lower as compared to other non-proliferating tissues and 100-fold lower compared to spleen. These results may help to explain tissue specific toxicity observed with nucleoside analogs used in HIV treatment as well as symptoms in inherited mitochondrial TK2 deficiencies.

1. Introduction

Treatment of HIV infection with nucleoside reverse transcriptase inhibitors (NRTIs) is associated with a number of metabolic complications, including myopathy, peripheral neuropathy, cardiomyopathy, hepatotoxicity, lactic acidosis and lipodystrophy syndrome [1,2]. Mitochondrial toxicity of NRTIs is believed to play a major role in these side effects, resulting in mitochondrial DNA depletion and a decreased oxidative phosphorylation activity [3–5]. It is still unknown why the toxicity of NRTIs specifically involves skeletal and heart muscles, liver, adipose tissue and peripheral nerves. Similarly, inherited mitochondrial DNA depletion syndrome caused by mutation(s) in the mitochondrial deoxynucleoside

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Abbreviations: AMV-RT, avian myeloblastosis virus reverse transcriptase; cN-1b, cytosolic 5′-nucleotidase 1b; cN-2, cytosolic 5′-nucleotidase 2; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; DNC, deoxynucleotide carrier; dNK, deoxynucleoside kinase; dNT-1, deoxynucleotidase 1; ecto-NT, ecto 5′-nucleotidase; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; HIV, human immunodeficiency virus; mt, mitochondria; NRTI, nucleoside reverse transcriptase inhibitor; NRTI-TP, NRTI triphosphate; 5′-dNT, 5′-nucleotidases; TK1, thymidine kinase 1; TK2, thymidine kinase 2

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kinases, i.e. TK2 and dGK, affected predominantly muscle (in the case of TK2), liver and brain (in the case of dGK mutation) [6,7]. Once inside the cells, NRTIs are phosphorylated to their monophosphates by cellular deoxynucleoside kinases (dNK), represented in the cytosol by TK1 and dCK, and in mitochondria by TK2 and dGK [8–10]. This is a rate-limiting step in the intracellular NRTI phosphorylation cascade and it is reversed by intracellular 5′-nucleotidases (5′-dNT) (cN1, cN2 and dNT1 in the cytosol, and dNT2 in mitochondria). Earlier studies indicate considerable overlap in the capacity to dephosphorylate nucleoside monophosphates between these enzymes, and their physiological role in natural and analog nucleotide metabolism is still under investigation [11,12]. Nevertheless, the relative activities of dNK and 5′-dNT are key factors in the control of the NRTI-TP pool sizes in the cytosol and mitochondria, particularly in tissues with resting cells with minimal *de novo* DNA precursor synthesis [9–12]. In addition, the deoxynucleotide carrier (DNC), a transporter of deoxynucleotides across the mitochondrial inner membranes, contributes to the mitochondrial DNA precursor pools in cells with significant cytosolic deoxynucleotide synthesis [13].

In order to clarify the mechanisms involved in the tissue specificity associated with NRTI toxicity and inherited TK2 and dGK deficiencies the expression profiles of cytosolic and mitochondrial dNKs and 5′-dNT as well as the DNC transporter were determined in mouse tissues, using Taqman real-time PCR and selective enzyme assay methods. The results presented here provide new information regarding the high variability in nucleotide metabolism in different tissues with important implications for the nucleoside analog toxicity, and DNA precursor turnover. This information is also important for the understanding of transgenic mice models affecting enzymes in DNA metabolism.

2. Materials and methods

2.1. Preparation of total tissues extracts

CD-1 mice (5–6 weeks old) were sacrificed and the tissues dissected and frozen by placing them in dry ice and stored at −80 °C. About 100 mg tissue was homogenized in 1 ml extraction buffer containing 50 mM Tris/HCl pH 7.6, 100 mM KCl, 2 mM DTT, 20% glycerol, 0.5% NP-40 and complete mini protease inhibitor cocktail (F. Hoffman-La Roche Ltd., Diagnostics Division, Basel, Germany) by using a Polytron device (PT 1200CL, Kinematica AG, Littau-Lucerne, Switzerland) at 4 °C. The homogenate was then subjected to three cycles of freezing and thawing, followed by sonication for 2× 15 s to release both cytosolic and mitochondrial proteins. The suspension was centrifuged for 20 min at 12,000 × *g* at 4 °C and the supernatant was stored in small aliquots at −80 °C. Protein concentration was determined by the Bradford method (BioRad Laboratories, Hercules, CA, USA) with BSA as standard protein.

2.2. Assays for deoxynucleoside kinase activities

The activities of the deoxynucleoside kinases were determined in total protein extracts by a radiochemical method [14]. In the standard assay 20–40 μg of total protein extract was incubated in reaction mixture, containing 50 mM Tris/HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM ATP, 10 mM NaF, 2 mM DTT, 0.5 mg/ml BSA and 22 μM [^{3}H]-dThd for total TK activity determinations; 22 μM [^{3}H]-dCyd plus 15 μM tetrahydrouridine (a cytidine deaminase inhibitor) for total dCK activity determinations; 43 μM [^{3}H]-dGuo plus 0.5 mM dCyd and 10 nM MDL 74428 (a PNP inhibitor [15] for selective dGK determinations; 105 μM [5-^{3}H]-Uridine for uridine kinase (EC 2.7.1.48) determinations. The TK1 and TK2 activities were calculated as described below. Since total TK activity is the sum of the TK1 and TK2 activities; the difference between total TK activity with and without 1 mM dCyd represent predominantly TK2 activity (which also use dCyd as substrate) present in the extract [10,14]. In case of total dGK activity it is the sum of the dGK and the dCK activities. However, the addition of 0.5 mM dCyd inhibited dCK more than 95% and give no significant effect on dGK the activity [8,14,16]. Activity measurements were repeated at least twice with each tissue extracts from 3 mice, and results are presented as mean ± S.D.

2.3. Assays for 5′-nucleotidases

The activity of dNT (i.e. both dNT1 and dNT2) [11,12] in crude extracts was measured using 0.2 mM [^{3}H]-dUMP (15 Ci/mmol, Moravek Biochemicals Inc., La Brea, CA, USA) in 25 mM acetate buffer, pH 5.5, 1 mM, methylene-ADP, 20 mM $MgCl_2$, 5 mM DTT, 30 mM KCl, and 0.2 mg/ml bovine serum albumin. Separation of labeled deoxyuridine from dUMP was performed by HPLC [14]. The activity of 5′-dNT was expressed as nmol/(mg min).

2.4. Enzyme assays for lactate dehydrogenase and citrate synthase

Citrate synthase (CS) and lactate dehydrogenase (LDH) activities were measured by spectrophotometric methods [14]. CS activity is expressed in μmol of mercaptide ion formed per 30 min per milligram of tissue and LDH activity is presented in μmol of NADH oxidized per milligram of tissue per 30 min. All activities were measured in duplicates of each tissue extracts from three mice and the results are presented as a mean ± S.D.

2.5. cDNA synthesis

Total RNA from mouse skeletal muscles, testis, brain, adipose tissue, liver, lung, kidney, heart and spleen was purchased from BioCat GmbH, Heidelberg, Germany. The cDNA was synthesized from 2 μg of total RNA in a reaction mixture containing 1 μg of random primers, 40 units of RNasin ribonuclease inhibitor, dNTPs (1 mM each) and 30 units of AMV RT (Promega GmbH, Mannheim, Germany) in a total volume of 25 μl. Two microliters of cDNA was used for each Taqman real-time PCR reaction.

2.6. Real-time quantitative PCR

Sequence specific primers and Taqman probes (MWG Biotech AG, Denmark) for nine genes were designed using Primer Express software (Applied Biosystem, Nordic Division, Stockholm, Sweden) and are shown in Table S1 (Supplemental

data). Equal amounts of cDNA from each sample were amplified in the reaction containing TaqMan Universal PCR master mix (Applied Biosystem) 900 nM primers and 250 nM Taqman probe in a total volume of 25 μl using the ABI PRISM 7700 thermocycler (Applied Biosystems) with the following conditions: 1 cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative amounts of deoxynucleoside kinase or 5′-nucleotidase mRNAs were calculated using the standard curve, according to User Bulletin #2 (ABI PRISM 7700 sequence detection system, Applied Biosystems). Standard curves for each gene were derived from pooled cDNAs, containing equal amounts of cDNA from mouse skeletal muscle, kidney, spleen, adipose tissue and testis. Relative levels of mRNA expression in tissues are presented in arbitrary units, derived from standard curves, per microgram of total RNA. The precision of this quantitation method was confirmed by similar results, obtained from two separate determinations of TK2, dGK and cN-1b mRNA levels.

2.7. *Statistics*

Statistical significance of correlations between activities and mRNA expression of deoxy)nucleoside kinases and dNT was analyzed using Sperman rank correlation.

3. Results

3.1. *mRNA expression of deoxynucleoside kinases, 5′-nucleotidases and DNC in mouse tissues*

The mRNA expression profiles of deoxynucleoside kinases and 5′-nucleotidases in nine mouse tissues were determined, with a focus on those tissues affected by NRTI-induced mitochondrial toxicity. A real-time Taqman PCR technique was used and initially the levels of GAPDH mRNA were intended to serve as references. However, we found a large tissue variation in GAPDH mRNA levels (Supplementary data, Fig. S1) and normalization of expression using GAPDH would affect the levels of these other genes in an unintended fashion [17]. For this reason the mRNA levels were expressed in arbitrary units, derived from standard curves, normalized per microgram of total RNA used in the reaction.

The levels of TK1 mRNA were barely detectable in brain, low in heart, skeletal muscle, adipose tissue, and six- to eight-fold higher in spleen and liver (Fig. 1). dCK expression was low in adipose tissue, two to four times higher in liver, heart and skeletal muscle, and six- to eight-fold higher in lung, brain, testis and spleen (Fig. 1). TK2 mRNA was low in heart, skeletal muscle, testis, and adipose tissue and three- to five-fold higher in liver, lung kidney, brain and spleen. There was less variation in dGK mRNA levels among the tissues, except for brain, lung, kidney, skeletal muscle and spleen the levels were two- to three-fold higher. DNC was expressed at similar and low levels in all tissues with the exception of kidney and skeletal muscle where levels were two- to three-fold higher (Fig. 1).

Kidney and brain had the highest expression of cN2, whereas in heart, adipose tissue, skeletal muscle and testis cN2 expression was four- to eight-fold lower (Fig. 1). dNT1 was expressed more uniformly across the tissues, except for about two-fold lower levels in heart and skeletal muscle. Expression of cN1b, the cN-1 gene identified in mouse tissues [18], was tissue-specific. It was absent in heart, liver and spleen, and extremely high in adipose tissue (73 times higher than in brain), relatively low in brain and testis, and very low in other tissues (Fig. 1). The most significant observation was that in the heart, adipose tissue and skeletal muscle all these genes are expressed at low levels compared to other tissues.

The very high level of cN1b mRNA found in adipose tissue is surprising and may be a PCR artifact, but the role of cN1b in adipose tissue is not known [12,18] and further studies are needed to verify this finding. Another unexpected observation was the high expression of the DNC transporter in mouse kidney and skeletal muscle. In the original report high levels of DNC mRNA were found in mouse kidney, lung, testis, spleen and brain [13]. The discrepancy noted here might be technical, since the real-time PCR technique used here had greater sensitivity than regular RT-PCR coupled with hybridization to the DNC radiolabeled probe and furthermore different region of the DNC mRNA were used as probes, 314–378 versus 110–131 in the first report [13]. In addition, DNC signal in the earlier report was normalized to β-actin mRNA amplified by RT-PCR. It is known that levels of β-actin vary among the different tissues and also that the comparison at the end point of PCR might affect the actual amounts of β-actin present in different tissues [17].

3.2. *Deoxynucleoside kinases and 5′-nucleotidases activities in mouse tissues*

Both the cytosolic and mitochondrial proteins were extracted by homogenization procedures, including the addition of detergents (0.5% NP-40). The levels of two marker enzymes, lactate dehydrogenase (LDH) representing cytosolic proteins

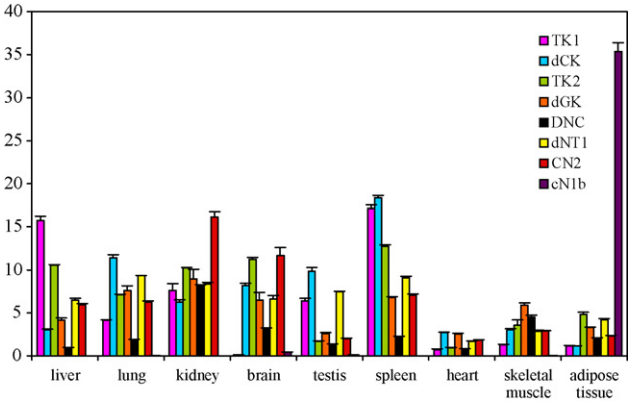

Fig. 1 – Expression of deoxynucleoside kinases, 5′-nucleotidases and DNC in mouse tissues. The mRNA levels of dNKs, DNC and 5′-dNTs in nine mouse tissues were quantified using a real-time Taqman PCR technique as described in materials and methods. mRNA expression is presented in arbitrary units, derived from the standard curves, per μg of total RNA used in the reaction, as a mean ± S.E. of two replicates from a single experiment or from two experiments (in the case of TK2 and dGK).

Table 1 – Specific activities of the deoxynucleoside kinases in mouse tissues extracts

Tissue	Thd	TK1:Thd + 1 mM dCyd	TK2	dCK:dCyd + 1 mM Thd	dGK:dGuo + 0.5 mM dCyd
Liver	4.4 ± 0.3	1.9 ± 0.1	2.5	4.4 ± 0.3	37.1 ± 5.8
Lung	3.3 ± 0.3	1.4 ± 0.1	1.9	3.7 ± 0.4	23.1 ± 2.3
Kidney	5.2 ± 0.7	2.1 ± 0.1	3.1	4.2 ± 0.6	36.6 ± 2.7
Brain	13 ± 1.0	4.8 ± 0.3	8.2	13 ± 1.0	15.9 ± 2.7
Testis	11 ± 0.8	6.4 ± 0.6	4.6	13 ± 0.9	8.1 ± 1.0
Pancreas	2.6 ± 0.3	1.3 ± 0.1	1.3	3.2 ± 0.2	7.8 ± 0.7
Spleen	211 ± 88	218 ± 89	–	56 ± 9.5	22.2 ± 4.0
Cardiac	1.9 ± 0.4	0.8 ± 0.1	1.1	1.2 ± 0.1	20.2 ± 1.0
Diaphragm	0.8 ± 0.1	0.3 ± 0.1	0.5	0.6 ± 0.1	14.0 ± 1.0
Biceps	0.4 ± 0.2	0.2 ± 0.1	0.2	0.4 ± 0.1	5.8 ± 1.5
Vastus	0.7 ± 0.4	0.1 ± 0.05	0.6	0.5 ± 0.4	5.1 ± 0.4
Semitend	0.5 ± 0.3	0.1 ± 0.05	0.4	0.4 ± 0.2	5.4 ± 0.9
Brown fat	5.7 ± 1.0	1.9 ± 0.2	3.8	5.1 ± 0.6	23.4 ± 1.1
Adipose	5.8 ± 3.0	2.4 ± 1.0	3.4	5.8 ± 2.8	20.7 ± 5.1

Assays were performed with ^{3}H-labeled deoxynucleosides as substrates (as indicated) in total mouse tissue extracts as described in materials and methods. Results are in pmol/(min mg) protein and presented as a mean ± S.D. of duplicate measurements of each tissue extracts from three mice.

and citrate synthase (CS) for mitochondrial proteins were determined in all the tissue extracts (Supplementary data, Table S2). The levels of LDH and CS varied about 10-fold between the extracts and the levels in extracts from epidymal fat tissue were the lowest for both enzymes. In case of LDH lung and pancreas extracts also had low levels, while activity in skeletal muscles was about 10-fold higher. The level of CS was also low in lung and pancreas, and additionally in the spleen. The highest CS activities were found in extracts from heart and brown fat. Taken together these results demonstrate considerable tissue-specific variation, related to the metabolic state of the tissues, of both LDH and CS, but all extracts contained significant levels of both enzymes indicating that both cytosolic and mitochondrial proteins were extracted.

The phosphorylation of Thd, dCyd and dGuo was determined in 14 different mouse tissues extracts in order to estimate the specific activities of TK1, TK2, dCK and dGK (Table 1). Extracts included different types of skeletal muscle with varying proportions of slow (type 1) and fast (type 2) fibers, e.g. Vastus intermedius with 35% type 2, Semitendinousus with more that 50% type 2, Diaphragm with 30% type 1 and 60% type 2 [19]. In addition, the levels of uridine kinase (UK) were also determined (Table 2). This ribonucleoside kinase appears to function in all cells and is a significant route of pyrimidine nucleotide synthesis in many tissues [20].

The actual phosphorylation rates measured in each case are shown in Table 1. Since deoxynucleoside kinases have overlapping substrate specificity data presented in Table 1 was the measurement of total activity. The activities of TK1, dCK, dGK can be relatively accurately determined by the remaining activities found when inhibitors of the interfering enzymes were added, e.g. the Thd phosphorylation in the presence of dCyd is to the major part due to TK1 (Table 1), the dCyd activities in the presence of Thd to dCK and the dGuo activities in the presence of dCyd due to dGK (Table 1). Determination of TK2 levels is problematic and has to rely on an indirect estimation based on the difference between the total TK values without and with addition of dCyd (Table 1), since dCyd is a substrate for TK2 but not for TK1 [9,10]. Due to the high TK1 activity in spleen extracts the TK2 level could not be accurately estimated in this case.

TK1 activities were very low in most tissues extracts except for testis and spleen. dCK activity was detected in all tissues but varied from 0.12 to 55 pmol/(min mg), with the lowest activity in muscle and the highest in spleen extracts. The levels of TK2 and dGK varied less (10–20-fold) but they were asymmetric in that TK2 levels were in most cases 10-fold lower than the dGK levels. The highest dGK activities were found in liver, kidney, lung, spleen, heart and brown fat and the lowest levels in muscle tissues. In all tissues, except spleen, brain and testis, dGK activities were two- to four-fold higher than any of the other deoxynucleoside kinases. The measurable TK2 levels were highest in brain, kidney, liver and brown fat and lowest in the biceps femoris muscle extracts (Table 1). There was no significant correlation between the TK2

Table 2 – Specific activities of uridine kinases and 5′-nucleotidase in mouse tissues extracts

Tissue	dNT	UK
Liver	49.3 ± 1.6	6.1 ± 1.2
Lung	147 ± 7.9	9.3 ± 0.7
Kidney	160 ± 3.2	16.1 ± 1.6
Brain	160 ± 2.9	19.1 ± 2.5
Testis	42.2 ± 1.7	16.0 ± 1.7
Pancreas	157 ± 3.5	5.5 ± 0.5
Spleen	504 ± 27	16.8 ± 2.5
Cardiac	70.0 ± 2.5	3.9 ± 0.6
Diaphragm	26.6 ± 1.0	4.4 ± 0.9
Biceps	12.8 ± 0.3	2.5 ± 0.2
Vastus	10.3 ± 1.0	2.6 ± 0.1
Semitend	16.8 ± 1.6	2.3 ± 0.2
Brown fat	145 ± 2.9	2.2 ± 0.2
Adipose	79.1 ± 1.9	11.2 ± 1.6

Assays were performed with ^{3}H-Urd and ^{3}H-dUMP as substrates in total mouse tissue extracts as described in materials and methods. Results are in pmol/(min mg) protein for UK and nmol/(min mg) for as 5′-dNT and presented as a mean ± S.D. of duplicate measurements of each tissue extracts from three mice.

and dGK activities observed in this study. The activities of UK were, in general, higher (about 10-fold) than those of the deoxynucleoside kinases and varied about 50-fold between the highest in spleen extracts (504 pmol/(min mg)) to the lowest in vastus intermedius extracts (10.3 pmol/(min mg)) (Table 2). There were significant correlations between the UK levels in the tissue extracts and those of TK1, dCK, dGK (ρ values 0.63–074 and p values 0.01–0.005) and only in case of TK2 activities there was no correlation with the UK levels.

The dNT activities were also measured using dUMP as substrate (Table 2). This activity is about 1000-fold higher than those of the deoxynucleoside kinases (i.e. nmol/(mg min) in comparison to pmol/(mg min)). The reason(s) for this large difference in activity is not known but may be due to the assay conditions used and it has been found consistently in many studies [14,21,22]. dNT activity varied approximately eight-fold between tissues, with brown fat as the lowest and kidney as the highest. The levels in spleen and brain were also high followed by those in testis, liver and lung, while the levels in adipose tissue and muscle extracts were four- to eight-fold lower (Table 2).

4. Discussion

NRTI treatment of HIV patients resulted in tissue specific mitochondrial toxicity, affecting muscles, adipose tissue, liver, peripheral sensory nerves, and heart (1–5). Several of these tissues are also selectively affected by inherited mtDNA depletion syndromes caused by mutations in mitochondrial deoxynucleoside kinases [6,7]. Mutation in dGK resulted in mtDNA depletion predominantly in liver and brain, whereas mutation in TK2 depleted the mtDNA content mainly in muscle [6,7]. One hypothesis is that certain tissues are more sensitive to disturbances in mtDNA precursor synthesis. Since in non-proliferating tissues the synthesis of mtDNA precursors is conducted *in situ* by the mitochondrial TK2 and dGK and there is very low level of nucleotide interchange between cytosol and mitochondria since the *de novo* synthesis is not active. Therefore, it is of importance to study the normal levels of dNK and 5′-dNT of cytosolic and mitochondrial origin as well as differences in the transport of deoxynucleotides across mitochondrial membrane via the DNC protein [13]. Here we presented the mRNA expression profiles of deoxynucleoside kinases, 5′-nucleotidases and DNC in nine mouse tissues, including those tissues affected by NRTI treatment or inherited mtDNA depletion syndromes. The results showed that each tissue has a unique expression pattern, with differences in the levels of cytosolic and mitochondrial deoxynucleoside kinases and 5′-nucleotidases.

A basic assumption in this study was that we have measured the expression of individual enzymes of importance for accumulation of nucleotides in cells in different tissues. Determination of the mRNA levels as well as activities of these enzymes will provide us an insight of DNA precursor synthesis in different tissues. Here the selectivity of the enzyme assays needs to be addressed since TK1, TK2, dCK and dGK as well as and 5′-nucleotidases have overlapping substrate specificities. In tissues with very asymmetric expression of the deoxynucleoside kinases, high levels of TK1 and dCK affect determinations of TK2 and dGK activities. This fact led to that in spleen extracts no TK2 levels could be estimated. Recently, a method for determining TK2 activities based on phosphorylation of the alternate substrate bromovinyl-deoxyuridine (BVDU) was published and it was shown to be a more selective as those described above [23]. The levels of TK2 in the mouse tissue extracts were measured using a modification of the BVDU method. The results showed a good correlation to those described here except in spleen and liver extracts (Wang and Eriksson, in preparation).

The assays for 5′-NT does not distinguished between the cytosolic dNT1 and the mitochondrial dNT2 activities [12,21,22], since the extracts contained enzyme from both compartments. There are selective inhibitors [21,22], which allow more precise measurements of the two enzymes but these were not used here. Furthermore, there is also some dephosphorylation of dUMP carried out by other 5′-nucleotidases (e.g. cN2) but the activity measured here should be predominantly due to dNT1 activity. There is a considerable overlap in the capacity of various 5′-dNT's to dephosphorylate deoxynucleotide analogs and it is very difficult to predict the *in vivo* contribution to degradation of nucleotide analogs by the various 5′-NT's [12,21,22]. However, the intention here was to get an estimate of pyrimidine deoxynucleotide dephosphorylation in various tissue extracts. In case of the purine deoxynucleotides the responsible 5′-NT is presently not known [12].

Our results demonstrate large variations in the mRNA and enzyme activity levels measured in mouse tissues. There was no correlation between the mRNA levels and the enzyme activities, although earlier studies have shown varying degrees of correlation between mRNA and activity for TK1 [8,9,14,24], and dCK [8,9,25–28]. One unexpected observation is the high level of TK1 mRNA found in liver without an increased TK1 enzyme activity. A similar phenomenon was previously described in chronic leukemia cells and indicates that in some situations high levels of apparently nonfunctional TK1 mRNA occur [24]. The RNA and protein extracts were not prepared from the same mice and this fact may at least in part account for this lack of correlation. However, there are to our knowledge no reports of mouse strain variation in enzymes in nucleoside metabolism. The results presented here and those referred to above are of importance since they indicate that it is not possible to use estimation of mRNA levels to predict the levels of enzyme activities in mouse tissues.

The levels of the dNK and 5′-dNT activities show a very large variation, demonstrating that the capacity to salvage pyrimidine deoxynucleosides is tissue specific with spleen being in a group of its own, followed by brown fat and testis, while the heart and skeletal muscles have a much lower capacity, irrespective of their differences in muscle fibers and concomitant variation in oxidative activity. The situation for purine deoxynucleoside salvage is different in that the levels of dGK do not vary to the same extent and most tissues possess relatively high dGK activity.

Taken together our data show that adipose tissue, heart and skeletal muscles have low levels of both the anabolic and catabolic nucleoside metabolizing enzymes that may explain the adverse side effects of NRTI therapy in these tissues. We

suggest that the mechanism of NRTI toxicity is due to disturbances in deoxynucleotide metabolism by the presence of nucleoside analogs and that adipose tissues, heart and skeletal muscles are very sensitive to this effect. There may not be a requirement that nucleoside analogs are incorporated in mtDNA in order to generate the deleterious effects. Consequently, NRTIs often inhibit normal mitochondrial dNTP turnover resulting in mtDNA damage and/or depletion. Interference with mtDNA synthesis and maintenance is therefore be a general risk factor in nucleoside analog chemotherapy, but an increased understanding of the specificities and properties of the enzymes involved should enable prescreening procedures that could decrease this risk. Recently, Lynx and McKee [29] have demonstrated that in rat heart and liver mitochondria AZT serves as a competitive inhibitor of TK2 and this is apparently directly linked to mitochondrial DNA depletion.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.03.029.

REFERENCES

[1] Carr A, Cooper DA. Adverse effects of antiretroviral therapy. Lancet 2000;356:1423–30.
[2] Vigouroux C, Gharakhanian S, Salhi Y, Nguyen TH, Chevenne D, Capeau J, et al. Diabetes, insulin resistance and dyslipidaemia in lipodystrophic HIV-infected patients on highly active antiretroviral therapy (HAART). Diabet Metab 1999;25:225–32.
[3] Brinkman K, Smeitink JA, Romijn JA, Reiss P. Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. Lancet 1999;354:1112–5.
[4] White AJ. Mitochondrial toxicity and HIV therapy. Sex Transm Infect 2001;77:158–73.
[5] Lewis W, Day BJ, Copeland WC. Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. Nat Rev Drug Discov 2003;2:812–22.
[6] Saada A, Shaag A, Mandel H, Nevo Y, Eriksson S, Elpeleg O. Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. Nat Genet 2001;29:342–4.
[7] Mandel H, Szargel R, Labay V, Elpeleg O, Saada A, Shalata A, et al. The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. Nat Genet 2001;29:337–41.
[8] Eriksson S, Wang L. The role of the cellular deoxynucleoside kinases in activation of nucleoside analogs used in chemotherapy. In: Chu CK, editor. Recent Adv in Nucleosides Chem and Chemotherap, Elsevier Science B.V; 2002. pp. 455–475.
[9] Eriksson S, Munch-Petersen B, Johansson K, Eklund H. Structure and function of cellular deoxynucleoside kinases. Cell Mol Life Sci 2000;59:1327–46.
[10] Wang L, Eriksson S. Cloning and characterization of full-length mouse thymidine kinase 2: the N-terminal sequence directs import of the precursor protein into mitochondria. Biochem J 2000;351:469–76.
[11] Rampazzo C, Gallinaro L, Milanesi E, Frigimelica E, Reichard P, Bianchi V. A deoxyribonucleotidase in mitochondria: involvement in regulation of dNTP pools and possible link to genetic disease. Proc Natl Acad Sci USA 2000;97:8239–44.
[12] Bianchi V, Spychala J. Mammalian 5′-nucleotidases. J Biol Chem 2003;278:46195–8.
[13] Dolce V, Fiermonte G, Runswick MJ, Palmieri F, Walker JE. The human mitochondrial deoxynucleotide carrier and its role in the toxicity of nucleoside antivirals. Proc Natl Acad Sci USA 2001;98:2284–8.
[14] Rylova SN, Albertioni F, Flygh G, Eriksson S. Activity profiles of deoxynucleoside kinases and 5′-nucleotidases in cultured adipocytes and myoblastic cells: insights into mitochondrial toxicity of nucleoside analogs. Biochem Pharmacol 2005;69:951–60.
[15] Weibel M, Balzarini J, Bernhardt A, Mamont P. Potentiating effect of (2-[2-[(2-amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)methyl]-phenyl]ethenyl)-phosphonic acid (MDL 74,428), a potent inhibitor of purine nucleoside phosphorylase, on the antiretroviral activities of 2′,3′-dideoxyinosine combined with ribavirin in mice. Biochem Pharmacol 1994;48:245–52.
[16] Wang L, Karlsson A, Arner ES, Eriksson S. Substrate specificity of mitochondrial 2′-deoxyguanosine kinase. Efficient phosphorylation of 2-chlorodeoxyadenosine. J Biol Chem 1993;268:22847–52.
[17] Suzuki T, Higgins PJ, Crawford DN. Control selection for RNA quantitation. Biotechniques 2000;29:332–7.
[18] Sala-Newby GB, Newby AC. Cloning of a mouse cytosolic 5′-nucleotidase-I identifies a new gene related to human autoimmune infertility-related protein. Biochim Biophys Acta 2001;1521:12–8.
[19] Pette D, Spamer C. Metabolic properties of muscle fibers. Fed Proc 1986;45:2910–4.
[20] Moyer JD, Malinowski N, Ayers O. Salvage of circulating pyrimidine nucleosides by tissues of the mouse. J Biol Chem 1985;260:2812–8.
[21] Rampazzo C, Mazzon C, Reichard P, Bianchi V. 5′-Nucleotidases: specific assays for five different enzymes in cell extracts. Biochem Biophys Res Commun 2002;293:258–63.
[22] Mazzon C, Rampazzo C, Scaini MC, Gallinaro L, Karlsson A, Meier C, et al. Cytosolic and mitochondrial deoxyribonucleotidases: activity with substrate analogs, inhibitors and implications for therapy. Biochem Pharmacol 2003;66:471–9.
[23] Franzolin E, Rampazzo C, Perez-Perez MJ, Hernandez AI, Balzarini J, Bianchi V. Bromovinyl-deoxyuridine: a selective substrate for mitochondrial thymidine kinase in cell extracts. Biochem Biophys Res Commun 2006;344:30–6.
[24] Kristensen T, Jensen HK, Munch-Petersen B. Overexpression of human thymidine kinase mRNA without corresponding enzymatic activity in patients with chronic lymphatic leukemia. Leuk Res 1994;18:861–6.
[25] Sigmond J, Kroep JR, Loves W, Codacci-Pisanelli G, Peters GJ. Quantitative real time PCR of deoxycytidine kinase mRNA by Light Cycler PCR in relation to enzyme activity and gemcitabine sensitivity. Cancer Lett 2004;213:173–9.

[26] Veuger MJ, Heemskerk MH, Honders MW, Willemze R, Barge RM. Functional role of alternatively spliced deoxycytidine kinase in sensitivity to cytarabine of acute myeloid leukemic cells. Blood 2002;99:1373–80.
[27] Lotfi K, Karlsson K, Fyrberg A, Juliusson G, Jonsson V, Peterson C, et al. The pattern of deoxycytidine- and deoxyguanosine kinase activity in relation to messenger RNA expression in blood cells from untreated patients with B-cell chronic lymphocytic leukemia. Biochem Pharmacol 2006;71:882–90.
[28] Arner ES, Flygar M, Bohman C, Wallstrom B, Eriksson S. Deoxycytidine kinase is constitutively expressed in human lymphocytes: consequences for compartmentation effects, unscheduled DNA synthesis, and viral replication in resting cells. Exp Cell Res 1988;178: 335–42.
[29] Lynx MD, McKee EE. 3′-Azido-3′-deoxythymidine (AZT) is a competitive inhibitor of thymidine phosphorylation in isolated rat heart and liver mitochondria. Biochem Pharmacol 2006;72:239–43.