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Biochemical and Biophysical Research Communications 344 (2006) 30–36
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Bromovinyl-deoxyuridine: A selective substrate for mitochondrial thymidine kinase in cell extracts

Elisa Franzolin [a], Chiara Rampazzo [a], María-Jesús Pérez-Pérez [b], Ana-Isabel Hernández [b], Jan Balzarini [c], Vera Bianchi [a,*]

[a] Department of Biology, University of Padova, 35131 Padova, Italy
[b] Instituto de Quimica Médica (CSIC), 28006 Madrid, Spain
[c] Rega Institute for Medical Research, K. U. Leuven, B-3000 Leuven, Belgium

Received 23 March 2006
Available online 31 March 2006

Abstract

Cellular models of mitochondrial thymidine kinase (TK2) deficiency require a reliable method to measure TK2 activity in whole cell extracts containing two interfering deoxyribonucleoside kinases, thymidine kinase 1 (TK1) and deoxycytidine kinase. We tested the value of the thymidine analog (E)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) as a TK2-specific substrate. With extracts of OSTTK1$^-$ cells containing TK2 as the only thymidine kinase and a highly specific TK2 inhibitor we established conditions to detect the low TK2 activity commonly present in cells. With extracts of TK1-proficient osteosarcoma cells and normal human fibroblasts we showed that BVDU, but not 1-(β-D-arabinofuranosyl)thymine (Ara-T), discriminates TK2 activity even in the presence of 100-fold excess TK1. A comparison with current procedures based on TK2 inhibition demonstrated the better performance of the new TK2 assay. When cultured human fibroblasts passed from proliferation to quiescence TK2 activity increased by 3-fold, stressing the importance of TK2 function in the absence of TK1.

Keywords: Thymidine kinase 2; Thymidine analogs; Mitochondrial DNA precursors; TK2-deficiency; Mitochondrial diseases; Mitochondrial DNA depletion syndromes; Enzyme assays; TK2 inhibitors

Genetic deficiency for the mitochondrial thymidine kinase (TK2) is the cause of a severe myopathic form of mitochondrial DNA depletion syndrome in humans [1]. This discovery has highlighted the lack of information on the physiological function of the enzyme and the need for methods to accurately measure TK2 activity in cell extracts for the screening of patients and the study of TK2 in experimental model systems. Human cells contain two thymidine kinases, cytosolic TK1 which is a powerful cell-cycle regulated enzyme [2–4], and TK2 which is expressed constitutively in all growth phases and tissues, at higher levels in mitochondria-rich tissues such as muscle and brain [5,6]. Both kinases phosphorylate thymidine and deoxyuridine but pure TK1 is 15- to 50-fold more efficient than pure TK2 [7]. In contrast to TK1, TK2 phosphorylates also deoxycytidine that is, however, the preferred substrate for the cytosolic deoxycytidine kinase (dCK) [8,9]. Besides participating in the salvage of natural deoxyribonucleosides, the kinases phosphorylate, with different efficiencies and specificities, nucleoside analogs used as antiviral and anticancer drugs [7,9]. Considering that the specificity of TK1 and TK2 for some analogs is more distinct than for the natural substrates [7], Arnèr et al. [10] suggested a system of assays to discriminate the activity of the two thymidine kinases present in whole cell extracts. They used AZT as the preferred TK1 substrate and AraT as the preferred TK2 substrate to evaluate which fraction of total TK activity approximately corresponds to TK2. An alternative approach is based on a specific inhibition of TK2. In the presence of the inhibitor the phosphorylation of

* Corresponding author. Fax: +39 049826280.
E-mail address: vbianchi@mail.bio.unipd.it (V. Bianchi).

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doi:10.1016/j.bbrc.2006.03.147

radioactive thymidine is catalyzed by TK1 and TK2 activity is calculated from the difference between the phosphorylation in the absence and presence of the inhibitor. We have used inhibition by BVDU, a highly specific and efficient alternative substrate of TK2 [11,12], to evaluate the activity of the mitochondrial kinase in human fibroblasts [13], others used deoxycytidine to inhibit TK2 [14]. Alternatively, phosphorylation of [^{3}H]deoxycytidine was measured in the presence and absence of thymidine to inhibit TK2 [15]. However, all these comparisons suffer from the fact that in cell extracts TK1 and dCK are generally more active than TK2 so that TK2 may account for a very small fraction of the total phosphorylation. Furthermore, dCK expression depends on the cell type and TK1 activity varies widely with the percentage of S-phase cells present in individual samples, adding a further source of error to the above procedures.

Here, we describe a new application of the thymidine analog BVDU that we use in radioactive form as a specific TK2 substrate to assay TK2 in whole cell extracts. We validate the assay in a TK1-deficient osteosarcoma cell line where TK2 is the only active thymidine kinase and then test it with TK1-containing extracts where TK1 is 100-fold more active than TK2. In order to demonstrate that the procedure allows to specifically measure TK2 even in such conditions, we use a new specific inhibitor of TK2, KIN109, structurally related to a family of acyclic nucleoside analogs that are potent inhibitors of the mitochondrial thymidine kinase [16,17]. By comparing the effects of KIN109 on BVDU phosphorylation in the different cell extracts we confirm that the specificity of BVDU for TK2 is sufficiently high to detect TK2 also in the presence of a large excess of TK1. We believe that this assay can be valuable for studies of TK2 expression in cultured cells and for the screening of patients.

Materials and methods

Materials. [*methyl*-^{3}H]Thymidine was from Perkin-Elmer Life and Analytical Sciences, Boston MA, and [5′-^{3}H]5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and [*methyl*-^{3}H (*N*)]thymine-1-β-D arabinofuranoside (AraT) were from Moravek Biochemicals, Brea, CA. Radioactive substrates were used in the assays at a specific activity of about 1000 cpm/pmol, with the exception of AraT, that at concentrations below 1 μM was used undiluted (sp. activity of about 5000 cpm/pmol).

Preparation of the TK2 inhibitor KIN109. 1-[6-[1,1-(Diphenyl)-1-(4-pyridyl)methoxy] hexyl]thymine (KIN109) (Fig. 1) was synthesized in three steps following a synthetic strategy very similar to that previously described for similar structures [17]. The commercially available diphenyl-4-pyridylmethanol (Aldrich, 75% technical grade) was transformed into the corresponding chloride following described procedures [18]. Next it was reacted with 1,6-hexanediol in the presence of Et_3N to afford 6-[1,1-(diphenyl)-1-(4-pyridyl)methoxy]hexanol. Incorporation of the thymine base was performed by a Mitsunobu-type condensation with N^3-benzoylthymine [17] followed by basic deprotection to afford KIN109. Full details of the analytical and spectroscopic properties of KIN109 will be reported elsewhere. (A.I. Hernández, O. Familiar, J. Balzarini, F. Rodríguez-Barrios, F. Gago, A. Karlsson, M.J. Camarasa, and M.J.Pérez-Pérez, unpublished results).

Cells and culture conditions. Human osteosarcoma cell lines OSTTK1$^-$ and HOSTK1$^+$ and the normal human lung fibroblast line CCD 34Lu were available in the laboratory and cultured as described earlier [13,19]. Proliferating cultures of CCD fibroblasts contained about 25% and quiescent cultures less than 1% S-phase cells as determined by flow-cytometric analysis.

Preparation of cell extracts. All manipulations were done close to 0 °C. Cell monolayers grown in 10 cm Petri dishes were rinsed three times with ice-cold 0.9% NaCl, carefully drained, and scraped off the dish in 200–300 μL of buffer containing 10 mM Tris–HCl, pH 7.5, 0.5% Triton X, 2 mM EDTA, and 1 mM DTT. The cell lysate was supplemented with 0.2 M NaCl, centrifuged 20 min at 19,000*g* at 4 °C and the supernatant was aliquoted and stored at −80 °C. Protein concentration was determined by the colorimetric procedure of Bradford [20] with bovine serum albumin as standard.

Assay conditions. All reactions were run with two different amounts of protein extract such that consumption of substrate did not exceed 15% during the incubation. Reactions were run in a total volume of 40 μL with 10–35 μg of protein (0.1 and 0.2 μg when HOSTK$^+$ extracts were assayed with [^{3}H]TdR) dissolved in 20 μL of 2× dilution buffer containing 1 mg/mL BSA, 10 mM Tris–HCl, 2 mM DTT, and 5 mM ATP, and added to 20 μL of a 2× reaction mix composed of 100 mM Tris–HCl, pH 7.5 (pH 8.0 with [^{3}H]AraT), 10 mM ATP, 10 mM $MgCl_2$, 1 mg/mL BSA, 10 mM NaF (20 mM with [^{3}H]AraT and [^{3}H]CdR), 4 mM DTT, and labelled substrate. With each concentration of substrate a parallel sample without protein was used as blank. The TK2 inhibitor KIN109 was dissolved in DMSO and added to 10% v/v in the reaction mix and the same % of DMSO was added to the reaction mixture of the controls. The presence of DMSO lowered by about 50% the phosphorylation of TdR and AraT and about 30% that of BVDU. To inhibit TK2 phosphorylation of [^{3}H]TdR unlabelled CdR was used at 500 or 1000 μM [14] and unlabelled TdR was used at 1000 μM to inhibit phosphorylation of 10 μM [^{3}H]CdR [15]. Reactions were incubated at 37 °C for different times with the different substrates: 20 min with [^{3}H]TdR, 40 min with [^{3}H]AraT, 60 min with [^{3}H]BVDU, and 20 and 40 min with [^{3}H]CdR. At the end of the incubation, 30 μL of the reaction volume was spotted on Whatman DE81 filters that were washed 3× for 5 min in 5 mM ammonium formate. Washing was done with a large excess of solvent, pooling together only filters with similar expected radioactivities, to avoid cross-contamination. Washed filters were eluted for 30 min with 2 mL of 0.1 M HCl, 0.2 M NaCl. In the assays with [^{3}H]AraT and [^{3}H]CdR elution was in 0.1 M HCl, 0.2 M KCl [10]. Radioactivity in the elution buffer was counted after adding 14 mL of Ready Safe scintillation fluid (Beckman Coulter, Fullerton, CA). We express here enzyme activity as pmol product/min/mg protein. In all graphs standard deviations of the means are shown only when they exceeded the sizes of the symbols on the curves.

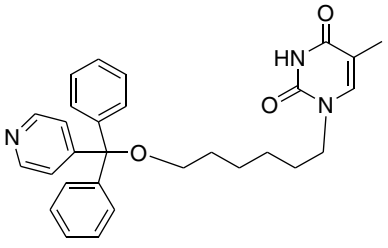

Fig. 1. Chemical structure of the TK2 inhibitor KIN109.

Results

To define a sensitive and specific assay for TK2 in whole cell extracts where the mitochondrial enzyme coexists with cytosolic TK1, we used [^{3}H]BVDU as a substrate on account of its several 100-fold higher affinity for TK2 than TK1 [11]. We first measured its phosphorylation in extracts from a human osteosarcoma cell line lacking TK1 and by means of a specific TK2-inhibitor established that the

reaction was specific for TK2. Thereafter we applied the assay to extracts from TK1-proficient cells to identify appropriate conditions to minimize the influence of TK1 and compared our method with the currently used procedures.

Comparison of TdR and BVDU phosphorylation in extracts of OSTTK1$^-$ cells

We used proliferating cultures of OSTTK1$^-$ cells as the source of crude cell extracts devoid of TK1 activity to measure the phosphorylation of increasing concentrations of [^{3}H]TdR and [^{3}H]BVDU by TK2 (Fig. 2). At all concentrations the rates of phosphorylation were low with both substrates, slightly higher with TdR than with BVDU at 0.2–5 μM. To ascertain that the activity depended on TK2, we incubated the extracts with [^{3}H]TdR in the presence and absence of 25 μM KIN109, a specific competitive inhibitor of TK2, and a highly effective inhibitor of the phosphorylation of 1 μM thymidine by purified recombinant TK2 ($IC_{50} = 0.47 \pm 0.03$ μM). In OSTTK1$^-$ cell extracts 25 μM KIN109 completely blocked the reaction at 0.2 and 1 μM TdR and produced a 70% inhibition at 10 μM TdR (data not shown). We then tested the effects of 25–100 μM KIN109 on the phosphorylation of [^{3}H]BVDU (Fig. 3). All concentrations of KIN109 inhibited the reaction strongly ($\geqslant$90%) at 0.2 μM BVDU, whereas at higher substrate concentrations the inhibition declined progressively. Thus in whole cell extracts KIN109 competed more effectively with the natural substrate TdR than with BVDU, but at 0.2 μM BVDU it completely blocked the reaction. This observation was used in the following experiments to evaluate TK2 contribution to the phosphorylation measured in TK1-proficient cells.

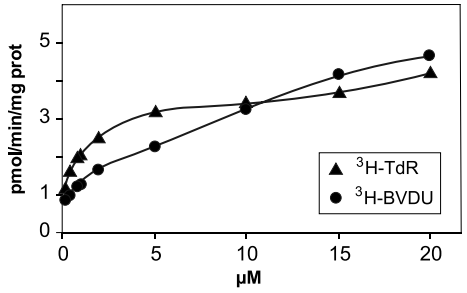

Fig. 2. Phosphorylation of [^{3}H]TdR and [^{3}H]BVDU in extracts from OSTTK1$^-$ cells. Protein extracts (17 and 34 μg) were incubated with increasing concentrations of [^{3}H]TdR (triangles) or [^{3}H]BVDU (circles).

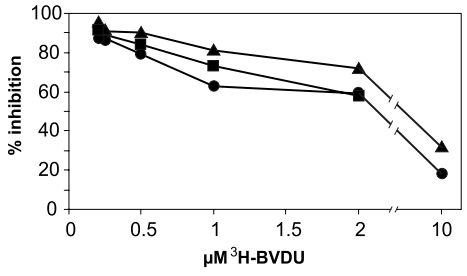

Fig. 3. Effects of the TK2 inhibitor KIN109 on the phosphorylation of [^{3}H]BVDU by extracts from OSTTK1$^-$ cells. Cell extracts (17 or 34 μg) were incubated for 60 min with the indicated concentrations of [^{3}H]BVDU in the absence or presence of 25 (circles), 50 (squares) or 100 (triangles) μM KIN109. The ordinate indicates % inhibition relative to the activity measured without KIN109.

Comparison of TdR and BVDU phosphorylation in extracts of TK1$^+$ cells

In order to assess the value of BVDU as a TK2-specific substrate, we compared the phosphorylation of [^{3}H]TdR and [^{3}H]BVDU by extracts from growing cultures of HOSTK1$^+$ cells containing both thymidine kinases but with TK1 activity greatly exceeding that of TK2 [21]. Now TdR phosphorylation was about 100-fold higher than BVDU phosphorylation (Fig. 4), in sharp contrast to the results with OSTTK1$^-$ extracts where only TK2 was present (Fig. 2). Low concentrations of BVDU gave similar specific activities for HOSTK1$^+$ and OSTTK1$^-$ extracts (Figs. 2 and 4B), whereas above 5 μM BVDU the specific activity in HOSTK1$^+$ extracts was 4- to 5-fold higher indicating a progressive engagement of TK1, the predominant thymidine kinase in HOS cells. Nevertheless also at substrate concentrations higher than 5 μM the total activity with BVDU was only about 2% of that measured with TdR as a substrate (Fig. 4A). To distinguish between the contribution of TK2 and TK1 we inhibited TK2 with 100 μM KIN109 at substrate concentrations between 0.25 and 2 μM (Table 1). The inhibitor showed only negligible effects on TdR phosphorylation indicating that TK1 was the responsible enzyme. With BVDU as substrate the

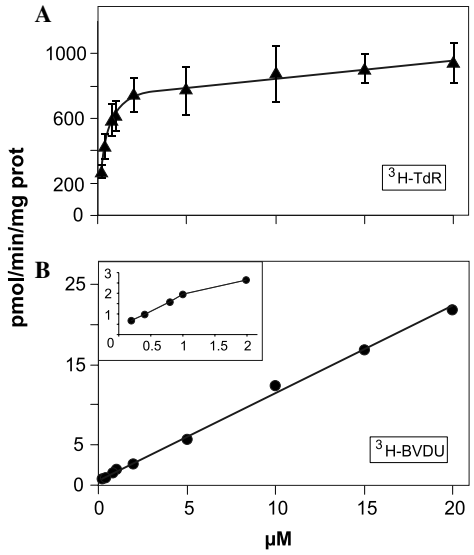

Fig. 4. Phosphorylation of [^{3}H]TdR and [^{3}H]BVDU in extracts from HOSTK1$^+$ cells. Extracts from proliferating cultures of HOSTK1$^+$ were incubated with labelled TdR (0.1 and 0.2 μg protein) (A) or BVDU (17 and 34 μg protein) (B) as in Fig. 1. The inset in B gives the activities at low concentrations of BVDU.

Table 1
Effects of 100 μM KIN109 on [^{3}H]BVDU and [^{3}H]TdR phosphorylation by extracts from growing HOSTK1$^+$ cells

Substrate (μM)	Activity (pmol/min/mg)		% Inhibition	Δ^* (pmol/min/mg)
	− KIN109	+ KIN109		
BVDU				
0.25	0.63	0.27	58	0.36
0.5	0.98	0.54	45	0.44
1	1.45	1.16	20	0.29
2	2.64	2.21	16	0.43
TdR				
0.25	118	102	13	16
1	345	310	10	35
2	466	431	7	35

Enzyme activity was measured with 0.1 and 0.2 μg protein for [^{3}H]TdR phosphorylation and with 17 and 34 μg for [^{3}H]BVDU phosphorylation. Values are means of duplicate determinations differing not more than 10% from the mean. Δ^*, difference between activity measured minus and plus KIN109.

reaction was more strongly inhibited by KIN109, although not to the extent observed in OSTTK1$^-$ cell extracts (Fig. 3). In TK1-deficient extracts 100 μM KIN109 inhibited the phosphorylation of 0.25 μM BVDU by 90% and that of 2 μM BVDU by 70% (Fig. 3), here at 0.25 μM BVDU the inhibition was 58%, suggesting that the phosphorylation largely occurred by TK2. Instead, at higher concentrations of BVDU the inhibitory effect of KIN109 became less pronounced indicating that now TK1 contributed more heavily to the activity. These results suggest that the phosphorylation of 0.25 μM BVDU can be used directly to measure TK2 activity with good approximation, even in the absence of a TK-2 inhibitor.

Until now we compared TdR and BVDU phosphorylation in extracts from two cancer cell lines, one devoid of TK1 activity, the other highly expressing TK1 during exponential growth. In order to test how cell proliferation influences the determination of TK2 activity, we applied the same assays in extracts from proliferating and quiescent cultures of human lung fibroblasts (CCD cells). Extracts from proliferating CCD cells showed a very high activity with TdR as substrate and a much lower activity with BVDU (Figs. 5A and B), similar to earlier results from HOSTTK1$^+$ cells. Extracts from quiescent cells showed, instead, similar and relatively low rates of phosphorylation of TdR and BVDU (Fig. 5C). The rates were, however, higher than those found with extracts from OSTTK1$^-$ cells (Fig. 2). KIN109 did not inhibit TdR phosphorylation in cycling cell extracts (Table 2) demonstrating that TK1 was the responsible enzyme. BVDU phosphorylation was more efficiently inhibited by KIN109 in these extracts than in extracts from HOSTK1$^+$ cells (Table 1), indicating that cycling CCD fibroblasts contain about 3- to 5-fold higher TK2 activity than HOS cells. Extracts from quiescent CCD cells showed a further 3-fold increase in TK2 activity relative to extracts from cycling cells, demonstrating that TK2 is the prevalent thymidine kinase during quiescence.

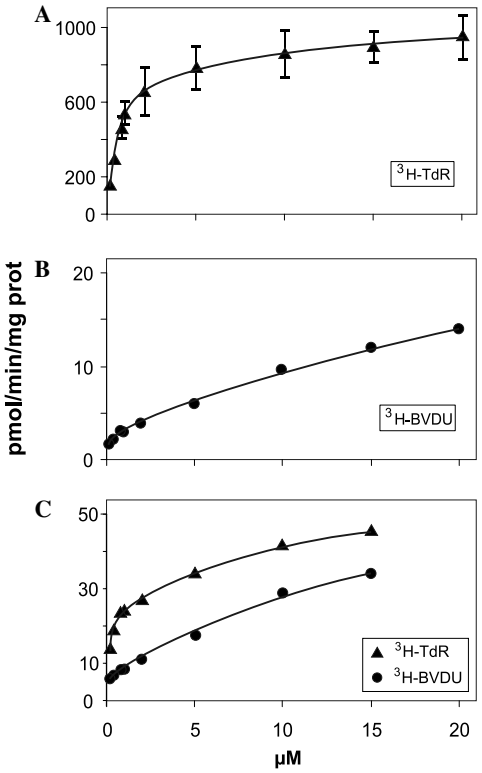

Fig. 5. Phosphorylation of [^{3}H]TdR and [^{3}H]BVDU in extracts from proliferating (A,B) and quiescent (C) cultures of CCD fibroblasts. Two different aliquots of protein extract, as in the Table 3, were used in each assay to maintain the consumption of substrate below 15% at all the concentrations tested.

AraT as substrate for TK2 and TK1 in cell extracts

We determined the effect of KIN109 on the phosphorylation of 0.2–100 μM [^{3}H]AraT by extracts of OSTTK1$^-$ and HOSTK1$^+$ cells (Fig. 6). In both cases the phosphorylation of AraT at less than 1 μM substrate concentration occurred at levels 10 times lower than those found with BVDU. The measurements required that AraT had the highest available specific radioactivity (about 5000 cpm/pmol). With only TK2 (OSTTK1$^-$ extracts) the activity reached a plateau at 50 μM AraT of about 2 pmol/min/mg and was completely inhibited by KIN109 (Fig. 6A). In the presence of both TK1 and TK2 (HOSTK1$^+$ cells) the activity increased almost linearly up to 100 μM AraT with a final value of 15 pmol/min/mg. In this case KIN109 had only a minor effect and inhibited the phosphorylation at most 30% at low concentrations of AraT (Fig. 6B). These results clearly show that AraT is phosphorylated also by TK1, especially at the high concentrations used earlier to assay TK2 [10].

The reciprocal TdR/CdR inhibition assay for TK2

An alternative approach to estimate TK2 activity in the presence of TK1 exploits the ability of deoxycytidine to serve as substrate for TK2 but not for TK1 [6,7]. One such assay measures [^{3}H]TdR phosphorylation in the absence or presence of an excess of unlabelled CdR assuming that

Table 2
Inhibition of [^{3}H]TdR and [^{3}H]BVDU phosphorylation by KIN109 in extracts from proliferating and quiescent CCD fibroblasts

Substrate (μM)	Activity (pmol/min/mg)		% Inhibition	Δ^* (pmol/min/mg)
	KIN109 25 μM			
	−	+		
Proliferating CCD				
BVDU				
0.2	1.5	0.2	87	1.3
1	2.7	1.1	59	1.6
10	11.1	9.7	13	1.4
TdR				
0.2	116	100	14	16
1	377	340	10	37
10	763	840	0	—
	KIN109 100 μM			
	−	+		
Quiescent CCD				
BVDU				
0.2	4.5	0.2	95	4.3
1	7.3	2.5	66	4.8
10	26	23	11	3
TdR				
0.2	5.2	1.0	81	4.2
1	16	3.1	81	12.9
10	33	11	67	22

In the case of proliferating cell extracts enzyme activity was measured with 0.1 and 0.2 μg protein for [^{3}H]TdR phosphorylation and with 17 and 34 μg for [^{3}H]BVDU phosphorylation, in the case of quiescent cell extracts with 2.6 and 5.2 μg ([^{3}H]TdR) and 1.3 and 2.6 μg ([^{3}H]BVDU). Values are means of duplicate determinations differing from the mean less than 10% for BVDU and less than 15% for TdR. Δ^*, difference between activity measured minus and plus KIN109.

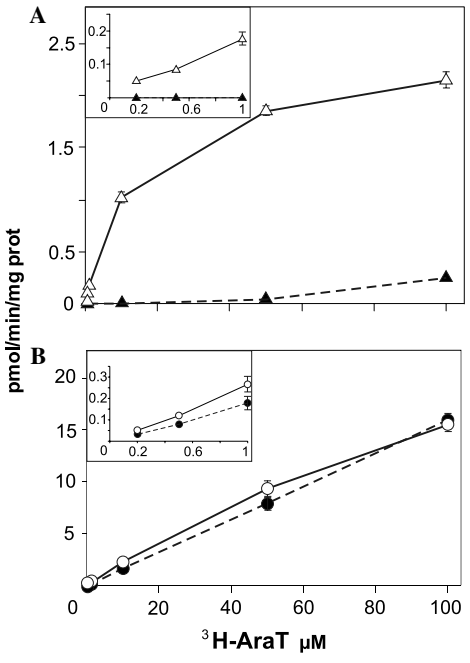

Fig. 6. Phosphorylation of 0.2–100 μM [^{3}H]AraT in extracts from OSTTK1$^-$ cells (A) and HOSTK1$^+$ cells (B) in the absence (empty symbols) or presence (filled symbols) of 100 μM KIN109. Extracts (15 and 30 μg protein) were incubated with [^{3}H]AraT as detailed under Materials and methods. Insets show results at submicromolar concentrations of substrate. Bars indicate the range of the determinations.

CdR blocks TK2 so that TdR phosphorylation depends on TK1. TK2 activity is then inferred from the difference in TdR phosphorylation under the two conditions [14]. With extracts of OSTTK1$^-$ cells we determined the effect of 0.5 and 1.0 mM CdR on the phosphorylation of 1 or 10 μM [^{3}H]TdR (Table 3). In all cases the inhibition was incomplete (at most 70%) in agreement with previous observations [14]. In extracts from HOSTK1$^+$ cells phosphorylation of 1 or 10 μM [^{3}H]TdR was so high relatively to that expected for TK2 that addition of CdR had no appreciable effect. From these results it appears that inhibition of TK2 by deoxycytidine does not provide a suitable method for the determination of TK2.

We also tested an alternative assay that measures [^{3}H]CdR phosphorylation in the presence or absence of an excess of unlabelled TdR that blocks TK2 [15]. In the presence of thymidine deoxycytidine is phosphorylated exclusively by dCK, in its absence both dCK and TK2 catalyze the reaction. At a concentration of 10 μM [^{3}H]CdR we found that OSTTK1$^-$ extracts phosphorylated deoxycytidine with a specific activity of 48 (pmol/min/mg protein) in the absence and 43 in the presence of 1 mM TdR. With HOSTK1$^+$ extracts we found a specific activity of 62 under both conditions. In both cases the activity of TK2 was clearly too low compared to that of dCK to calculate TK2 activity from the difference between the non-inhibited and inhibited reactions.

Table 3
Influence of excess unlabelled CdR on the phosphorylation of [^{3}H]TdR in extracts of OSTTK1$^-$ and HOSTK1$^+$ cells

[^{3}H]Substrate	Unlabelled substrate	OSTTK1$^-$ cells		HOSTK1$^+$ cells	
		Activity (pmol/min/mg)	% Inhibition	Activity (pmol/min/mg)	% Inhibition
1 μM TdR	CdR				
	—	2.42 ± 0.22	—	408 ± 71	—
	500 μM	0.87 ± 0.14	64	420 ± 51	—
	1000 μM	0.65 ± 0.10	70	454 ± 62	—
10 μM TdR	—	4.24	—	829	—
	500 μM	2.43	43	779	6
	1000 μM	2.24	47	747	10

Assays were run in duplicate with 17 and 34 μg proteins of OSTTK1$^-$ extract and 0.1 and 0.2 μg proteins of HOSTK1$^+$ extract in the [^{3}H]TdR reactions. Values from 2 experiments with 1 μM TdR and 1 with 10 μM TdR are the means of replicate values (±standard deviations in the case of 1 μM TdR).

Discussion

Determination of TK2 activity in cell extracts is complicated by interference from TK1 and dCK. BVDU is the nucleoside analog with the most strikingly different substrate activity between TK2 and TK1 [12] and it is commercially available in radioactive form. It appeared to be a suitable preferential TK2 substrate with sufficiently high affinity for the enzyme that it can be used at low concentrations without interference by TK1. Crucial for the development of the present assay was the availability of cells devoid of TK1 and the availability of KIN109, a new specific non-substrate inhibitor of TK2. In extracts from OSTTK1$^-$ cells [^{3}H]BVDU and [^{3}H]TdR were phosphorylated at very similar rates (Fig. 2). Both reactions were inhibited by KIN109 showing that TK2 was indeed responsible for the phosphorylation of BVDU (Fig. 3). On this basis we examined the influence of TK1 on BVDU phosphorylation in TK1-proficient cells. We could measure TK2 activity at submicromolar concentrations of BVDU even when the enzyme represented a minute fraction of total thymidine kinase activity in the cells (Figs. 4 and 5). Under similar conditions AraT was mostly phosphorylated by TK1 (Fig. 6B) and the currently used procedures that evaluate TK2 activity as the difference between TdR (or CdR) phosphorylation in the absence or presence of a TK2 inhibitor were inadequate (Table 3). Furthermore, the new assay demonstrated that the level of TK2 increases when human fibroblasts pass from the proliferative to the quiescent state (Figs. 5B and C), suggesting upregulation of TK2 function in quiescent cells that lack TK1 [2] and have very low levels of de novo synthesis of deoxyribonucleotides [22].

The use of the specific TK-2 inhibitor KIN109 was important here to detect the involvement of TK1 in the phosphorylation of substrates and establish the appropriate conditions to assay TK2 activity with BVDU. However, we do not think that a TK2 inhibitor is indispensable for a standard assay. On the basis of our present results we conclude that 0.2 μM [^{3}H]BVDU at a specific radioactivity of about 1000 cpm/pmol is a suitable substrate to measure TK2 activity in cell extracts. Some minor interference by TK1 may still occur when the activity of the cytosolic thymidine kinase in the extracts exceeds that of TK2 several 100-fold (Table 1). If in parallel assays with [^{3}H]TdR and [^{3}H]BVDU the values with TdR and BVDU are similar, as observed in Fig. 2 with OSTTK1$^-$ cells and in Fig. 5C with quiescent human fibroblasts, one can assume that all the activity observed with [^{3}H]BVDU is TK2. If the specific activity with [^{3}H]TdR as a substrate is much higher than that with [^{3}H]BVDU (Figs. 3, 5A and B), the latter value still approximates well the actual TK2 activity in the sample. For a more precise determination one can resort to the use of a specific TK2 inhibitor like KIN109.

In our opinion the above strategy is a substantial improvement over the current methods and we are confident that it may be helpful in studies of TK2 function. Only by this procedure could we detect silencing of TK2 enzymatic activity in cells where TK2 mRNA was downregulated by RNA interference (Rampazzo and Bianchi, unpublished).

Acknowledgments

The authors thank Peter Reichard for his suggestions and constructive criticism.This work was supported by grants from AIRC, Italian Association for Cancer Research, Italian Telethon (Grant GGP05001) and the Italian Ministry of Education and Research (Prin Project 2005) to V.B., the University of Padova to C.R., the Spanish MEC (SAF2003-07219-C02-01) to M.J.P-P., and the EU (QLRT- CT-2001-01004). A.I.H. thanks the Spanish Ministerio de Educación y Ciencia for a predoctoral fellowship.

References

[1] A. Saada, A. Shaag, H. Mandel, Y. Nevo, S. Eriksson, O. Elpeleg, Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy, Nat. Genet. 29 (2001) 342–344.
[2] B. Munch-Petersen, G. Tyrsted, Induction of thymidine kinases in phytohaemagglutinin-stimulated human lymphocytes, Biochim. Biophys. Acta 478 (1977) 364–375.

[3] J. Sherley, T. Kelly, Regulation of human thymidine kinase during the cell cycle, J. Biol. Chem. 263 (1988) 8350–8358.
[4] P.Y. Ke, Z.F. Chang, Mitotic degradation of human thymidine kinase 1 is dependent on the anaphase-promoting complex/cyclosome-CDH1-mediated pathway, Mol. Cell. Biol. 24 (2004) 514–526.
[5] A.J. Berk, D.A. Clayton, A genetically distinct thymidine kinase in mammalian mitochondria. Exclusive labeling of mitochondrial deoxyribonucleic acid, J. Biol. Chem. 248 (1973) 2722–2729.
[6] M. Johansson, A. Karlsson, Cloning of the cDNA and chromosome localization of the gene for human thymidine kinase 2, J. Biol. Chem. 272 (1997) 8454–8458.
[7] B. Munch-Petersen, L. Cloos, G. Tyrsted, S. Eriksson, Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides, J. Biol. Chem. 266 (1991) 9032–9038.
[8] C. Bohman, S. Eriksson, Deoxycytidine kinase from human leukemic spleen: preparation and characteristics of homogeneous enzyme, Biochemistry 27 (1988) 4258–4265.
[9] E.S. Arnèr, S. Eriksson, Mammalian deoxyribonucleoside kinases, Pharmacol. Ther. 67 (1995) 155–186.
[10] E.S. Arner, T. Spasokoukotskaja, S. Eriksson, Selective assays for thymidine kinase 1 and 2 and deoxycytidine kinase and their activities in extracts from human cells and tissues, Biochem. Biophys. Res. Commun. 188 (1992) 712–718.
[11] M. Johansson, A.R. Van Rompay, B. Degreve, J. Balzarini, A. Karlsson, Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of *Drosophila melanogaster*, J. Biol. Chem. 274 (1999) 23814–23819.
[12] J. Balzarini, C. Zhu, E. De Clercq, M.J. Perez-Perez, C. Chamorro, M.J. Camarasa, A. Karlsson, Novel ribofuranosylnucleoside lead compounds for potent and selective inhibitors of mitochondrial thymidine kinase-2, Biochem. J. 351 (2000) 167–171.
[13] P. Ferraro, G. Pontarin, L. Crocco, S. Fabris, P. Reichard, V. Bianchi, Mitochondrial deoxynucleotide pools in quiescent fibroblasts: a possible model for mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), J. Biol. Chem. 280 (2005) 24472–24480.
[14] L. Wang, S. Eriksson, Cloning and characterization of full-length mouse thymidine kinase 2: the N-terminal sequence directs import of the precursor protein into mitochondria, Biochem. J. 351 (2000) 469–476.
[15] S.N. Rylova, F. Albertioni, G. Flygh, S. Eriksson, Activity profiles of deoxynucleoside kinases and 5′-nucleotidases in cultured adipocytes and myoblastic cells: insights into mitochondrial toxicity of nucleoside analogs, Biochem. Pharmacol. 69 (2005) 951–960.
[16] J. Balzarini, A.I. Hernandez, P. Roche, R. Esnouf, A. Karlsson, M.J. Camarasa, M.J. Pérez-Pérez, Non-nucleoside inhibitors of mitochondrial thymidine kinase (TK-2) differentially inhibit the closely related herpes simplex virus type 1 TK and *Drosophila melanogaster* multifunctional deoxynucleoside kinase, Mol. Pharmacol. 63 (2003) 263–270.
[17] A.I. Hernández, J. Balzarini, A. Karlsson, M.J. Camarasa, M.J. Pérez-Pérez, Acyclic nucleoside analogues as novel inhibitors of human mitochondrial thymidine kinase, J. Med. Chem. 45 (2002) 4254–4263.
[18] S. Coyle, A. Hallett, M.S. Munns, G.T. Young, Amino-acids and peptides 45. The protection of the thiol function of cysteine and the imidazole-N of histidine by the diphenyl-4-pyridylmethyl group, J. Chem. Soc. Perkin Trans. 1 (1981) 522–528.
[19] C. Rampazzo, P. Ferraro, G. Pontarin, S. Fabris, P. Reichard, V. Bianchi, Mitochondrial deoxyribonucleotides, pool sizes, synthesis, and regulation, J. Biol. Chem. 279 (2004) 17019–17026.
[20] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[21] G. Pontarin, L. Gallinaro, P. Ferraro, P. Reichard, V. Bianchi, Origins of mitochondrial thymidine triphosphate: dynamic relations to cytosolic pools, Proc. Natl. Acad. Sci. USA 100 (2003) 12159–12164.
[22] P. Håkansson, A. Hofer, L. Thelander, Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells, J. Biol. Chem. (2006), doi:10.1074/jbc.M512894200.