Novel ribofuranosylnucleoside lead compounds for potent and selective inhibitors of mitochondrial thymidine kinase-2

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The ribonucleoside analogues (*E*)-5-(2-bromovinyl)uridine (5-BV-Urd) and 3'-spiro-(4"-amino-1",2"-oxathiole-2",2"-dioxide)-5-methyluridine (3'-AOD-5-MeUrd) emerged as potent and selective competitive inhibitors of mitochondrial thymidine kinase (TK)-2 with respect to thymidine (K_i/K_m values of 9.0 and 1.2 respectively). Cytosolic TK-1 did not show measurable affinity for these compounds. [³²P]Phosphate transfer studies from [γ -³²P]ATP to 5-BV-Urd and 3'-AOD-5-MeUrd revealed ex-

tremely poor substrate activity but potent inhibitory potential of the compounds. It was concluded that the ribonucleosides 5-BV-Urd and 3'-AOD-5-MeUrd represent two new lead compounds for potent and selective inhibitors of mitochondrial TK-2.

Key words: antiviral compounds, chemotherapy, cytostatic compounds, drugs, mitochondria.

INTRODUCTION

In mammalian cells, there are two deoxyribonucleoside kinases that are able to phosphorylate thymidine, namely cytosolic thymidine kinase (TK)-1 and mitochondrial thymidine kinase, TK-2 [1]. Both enzymes are genetically distinguishable isoenzymes. TK-1 is present in the cytosol of almost all types of organisms and is tightly regulated during the cell cycle. Both TK-1 mRNA and protein rise near the G1/S boundary, peak in early S-phase, and decline again in the G2-phase [2,3]. TK-1 phosphorylates the natural substrates 2'-deoxythymidine (dThd) and 2'-deoxyuridine (dUrd). TK-2 is a mitochondrial enzyme [4]: it represents a rather small fraction of cellular TK levels, compared with TK-1 activity, in proliferating cells. However, TK-2 corresponds to the predominant fraction of TK activity in resting or terminally differentiated cells (i.e. monocytes/macrophages) where TK-1 activity is virtually undetectable. The constitutive expression of TK-2 is most probably correlated with the mitochondrial content of the tissues rather than with the growth state of the cells. Like cytosolic TK-1, human mitochondrial TK-2 efficiently phosphorylates dThd and dUrd, but, in contrast with TK-1, TK-2 also efficiently recognizes 2'-deoxycytidine (dCyd) as a substrate [4–6].

The natural ribonucleosides uridine and cytidine are not substrates for TK-1. Thymine riboside has been reported to have a very poor substrate activity for TK-1 and TK-2 (approx. 2–3 % of dThd), and uridine was found to be a very poor substrate for TK-2 and not a substrate at all for TK-1 [7]. However, there are striking differences in substrate affinities of several biologically important nucleoside analogues for TK-1 and TK-2. Whereas 3'-azido-2',3'-dideoxythymidine ('AZT') is an excellent substrate for TK-1 and a poor substrate for TK-2, 1- β -D-arabinofuranosylthymine (araT) proved a much better substrate for TK-2 than for TK-1 [8], and araT has been used to discriminate between TK-1 and TK-2 activities in cell extracts. However, the most striking example of differential substrate activity between TK-1 and TK-2 is undoubtedly represented by the antihyper-

drug (*E*)-5-(2-bromovinyl)-dUrd (BVDU), which lacks any measurable substrate affinity for TK-1, but is an excellent substrate for TK-2 [9]. In contrast the anti-human immunodeficiency virus drug 3'-fluoro-2',3'-dideoxythymidine has been claimed to lack any substrate activity for TK-2 but is an excellent substrate of TK-1 [6,10]. The fact that TK-2 recognizes arabinosylpyrimidine nucleosides such as araT and the anti-hepatitis B virus agent 2'-fluoro-5-iodo-(1- β -D-arabinofuranosyl)uracil (FIAU) indicates that the arabinosyl configuration in the 2'-position of the furanose (i.e. OH, F) is not a hindrance for the phosphorylation of such nucleoside analogues by mitochondrial TK-2 [9].

In the present study we report the surprising finding that, in contrast with TK-1, TK-2 can be markedly inhibited by ribofuranosylnucleoside analogues. These findings may have important implications in the search for efficient and selective substrates and/or inhibitors of TK-2 as novel anticancer or antimetabolic agents.

MATERIALS AND METHODS

Compounds

Thymidine, uridine, cytidine, 5-fluorouridine, 5-iodouridine and 5-methyluridine were purchased from Sigma (St. Louis, MO, U.S.A.). BVDU was synthesized according to a previously described procedure [11]. (*E*)-5-(2-bromovinyl)uridine (5-BV-Urd) was synthesized as described previously [12], and the synthesis of 3'-spiro-(4"-amino-1",2"-oxathiole-2",2"-dioxide)-5-methyluridine (3'-AOD-5-MeUrd) has previously been reported [13,14].

Enzyme

Human mitochondrial TK-2 cDNA was inserted into the pGEX-5X-1 plasmid vector, expressed as a fusion protein to glutathione

Abbreviations used: TK, thymidine kinase; 5-BV-Urd, (*E*)-5-(2-bromovinyl)uridine; 3'-AOD-5-MeUrd, 3'-spiro-(4"-amino-1",2"-oxathiole-2",2"-dioxide)-5-methyluridine; BVDU, (*E*)-5-(2-bromovinyl)-2'-deoxyuridine; araT, 1- β -D-arabinofuranosylthymine; FIAU, 2'-fluoro-5-iodo-(1- β -D-arabinofuranosyl)uracil; dThd, 2'-deoxythymidine; dUrd, 2'-deoxyuridine; dCyd, 2'-deoxycytidine.

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S-transferase, and purified as previously described [4]. Briefly, the expression plasmid vector was transformed into the *Escherichia coli* strain BL21 (DE3) pLysS (Stratagene). A transformed colony was inoculated in Luria–Bertani medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The bacteria were grown to $D_{600} = 0.7$ and protein expression was induced with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside for 4 h. The cells were harvested by centrifugation at 6000 *g* for 10 min and were resuspended in PBS. The bacteria were lysed by sonication for 10 min on ice, and the extract was centrifuged at 10000 *g* for 10 min. The crude extract was loaded on to a GSH–Sepharose 4B column (Pharmacia), and the recombinant protein was eluted in 100 mM Tris (pH 7.6) supplemented with 10 mM GSH (Sigma). The size and purity of the recombinant protein were determined by SDS/PAGE (Phase System, Pharmacia).

Radiochemicals

The radiolabelled substrate [*Me*-³H]dThd (70 Ci/mmol) was obtained from Moravsek Biochemicals (Brea, CA, U.S.A.). [γ -³²P]ATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech.

TK assay using [*Me*-³H]dThd as the substrate

The activity of purified TK-1 [from human T lymphocyte (CEM) cells] and recombinant TK-2 was assayed in a 50-µl reaction mixture containing 50 mM Tris/HCl (pH 8.0), 2.5 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM CHAPS, 3 mg/ml BSA, 2.5 mM ATP and the indicated concentrations of [*Me*-³H]dThd and enzyme. The samples were incubated at 37 °C for 30 min in the presence or absence of different concentrations of the test compounds. Aliquots of 45 µl of the reaction mixtures were spotted on to Whatman DE-81 filter paper disks. The filters were washed three times for 5 min each in 1 mM ammonium formate, once for 1 min in water, and once for 5 min in ethanol. The radioactivity was determined by scintillation counting. The K_m , K_i and V_{max} values were derived from Lineweaver–Burk plots.

Phosphoribosyl transfer assay using [γ -³²P]ATP as the phosphate donor

The phosphoryl transfer assay was performed using [γ -³²P]ATP as the phosphate donor. The ribonucleosides were added to a final concentration of 5 µM and 100 µM in a 10-µl reaction mixture containing 50 mM Tris (pH 8.5), 5 mM MgCl₂, 1 mM unlabelled ATP, 100 µCi of [γ -³²P]ATP and 1 µg of recombinant TK-2. The samples were incubated for 30 min at 37 °C. Aliquots (2 µl) of the reaction mixtures were spotted on to polyethyleneimine-cellulose F TLC sheets (Merck), and the ribonucleosides were separated in a buffer containing NH₄OH/isobutyric acid/distilled H₂O (1:66:33, by vol.). The sheets were autoradiographed using phosphorimaging plates (BAS 1000, Fujix).

RESULTS

Inhibitory activity of a variety of ribofuranosyl nucleoside derivatives on TK-1- and TK-2-catalysed phosphorylation of [*Me*-³H]dThd

Uridine, cytidine and a series of 5-substituted furanosyl pyrimidine nucleoside analogues, including a 3'-spiro-substituted derivative (Table 1), have been evaluated for their inhibitory activity against phosphorylation of 2 µM dThd by purified

Table 1 Inhibitory activity of ribofuranosyluracil nucleoside analogues on TK-1- and TK-2-catalysed phosphorylation of [*Me*-³H]dThd

IC₅₀ or compound concentration required to inhibit phosphorylation of 2 µM [*Me*-³H]dThd by purified cytosolic TK-1 and mitochondrial recombinant TK-2.

Compound	IC ₅₀ (µM)	
	TK-1	TK-2
Uridine	> 1000	> 1000
Cytidine	> 1000	> 1000
5-F-Urd	> 1000	≥ 1000
5-I-Urd	> 1000	353 ± 216
5-Me-Urd	> 1000	148 ± 26
5-BV-Urd	> 1000	19 ± 1.1
3'-AOD-5-MeUrd	> 1000	4.6 ± 0.35
BVDU	> 1000	0.34

Figure 1 Structural formulae of test compounds that are recognized by mitochondrial TK-2

dThd, dUrd and dCyd are the known natural substrates of the enzyme. BVDU and araT are reported as selective substrates for the enzyme. 5-BV-Urd and 3'-AOD-5-MeUrd are novel selective inhibitors of TK-2 reported in the present study.

cytosolic TK-1 and recombinant mitochondrial TK-2. None of the compounds showed appreciable inhibition against TK-1 at 1000 µM. However, whereas uridine, cytidine and 5-fluorouridine had no measurable inhibitory effect on TK-2, 5-iodouridine and 5-methyluridine (ribothymidine) were slightly inhibitory (IC₅₀: 148–353 µM). The 5-BV-Urd (Figure 1) derivative markedly

Figure 2 Lineweaver–Burk plots for mitochondrial TK-2 in the presence of 5-BV-Urd (A) and 3'-AOD-5-MeUrd (B), with [*Me*-³H]dThd as the natural (competing) substrate

(A) Inhibitor concentrations are 50 μM ($\blacklozenge$), 25 μM ($\blacksquare$), 10 μM ($\blacktriangle$) and 0 μM ($\bullet$). (B) inhibitor concentrations are 5 μM ($\blacklozenge$), 2.5 μM ($\blacksquare$) and 0 μM ($\bullet$).

interfered with the TK-2-catalysed dThd phosphorylation, resulting in an IC_{50} of 19 μM , and, the 3'-AOD-5-MeUrd derivative of 5-methyluridine (Figure 1) was inhibitory at a concentration as low as 4.6 μM . BVDU, known as being an excellent substrate for TK-2 [15], inhibited the reaction at 0.34 μM [9].

Kinetic analysis of 5-BV-Urd and 3'-AOD-5-MeUrd against mitochondrial TK-2

The most potent inhibitors of TK-2 derived from the present study, i.e. 5-BV-Urd and 3'-AOD-5-MeUrd, were subjects of detailed kinetic analysis with regard to the nature of their inhibitory effect on mitochondrial TK-2. Each compound was added at two concentrations to a variety of [*Me*-³H]dThd concentrations ranging between 0.4 and 5 μM . Both compounds emerged as purely competitive inhibitors of the TK-2-catalysed dThd phosphorylation, as is evident from the Lineweaver–Burk graphs plotting the reciprocal of the reaction velocity versus the reciprocal of the dThd concentrations (Figure 2). 5-BV-Urd had a K_i of 10.4 μM and a K_i/K_m value of 9.0. The 3'-AOD-5-MeUrd derivative had a K_i as low as 1.4 μM and the K_i/K_m value was 1.2. Thus both compounds, particularly the 3'-AOD-5-MeUrd derivative, were endowed with a K_i value in the lower micromolar

Figure 3 Phosphorylation of dThd, BVDU, 5-BV-Urd and 3'-AOD-5-MeUrd by mitochondrial TK-2 in a [γ -³²P]ATP phosphate transfer assay from [γ -³²P]ATP

The compounds were exposed to TK-2 at 5 μM and 100 μM . The upper spots represent the formation of the [γ -³²P]monophosphate compounds. The spots at the bottom (start) of the thin-layer chromatogram represent [γ -³²P]ATP. The nature of the middle radiolabelled spots is unclear. It does not represent a reaction product derived from the test compounds since it is also present in a control experiment in which the compound was absent (2nd lane from the left). The spots may represent protein-[γ -³²P]ATP adducts or protein-[γ -³²P]-radiolabelled adducts, since the control assay in which [γ -³²P]ATP was run in the absence of both the compounds and the enzyme, showed a significantly reduced radiolabelled spot (first lane from the left). This minor radiolabelled spot may represent a radiolabelled adduct to the BSA that is present in the reaction mixture.

range. The K_i of 3'-AOD-5-MeUrd was comparable with the K_m value of the natural substrate dThd for mitochondrial TK-2.

Phosphate transfer from [γ -³²P]ATP to the test compounds by TK-2

To reveal whether 5-BV-Urd and 3'-AOD-5-MeUrd acted as substrates or inhibitors of the TK-2-catalysed nucleoside phosphorylation reaction, TK-2-catalysed [γ -³²P]ATP to the test compounds were performed. The natural substrate dThd and the well-known substrate BVDU were included as controls (Figure 3). Whereas both dThd and BVDU were extremely well-converted into their (radiolabelled) 5'-monophosphate derivatives (upper spots on the thin-layer chromatograms of Figure 3), 5-BV-Urd was poorly converted into 5-BV-UMP (weak upper spot). Moreover, 3'-AOD-5-MeUrd that had an enzyme affinity that was approx. 8-fold higher than that of 5-BV-Urd, was not measurably converted to its corresponding 5'-monophosphate derivative. 3'-AOD-5-MeUrd at either 5 μM or 100 μM failed to show formation of a visible radiolabelled spot which may have pointed to its conversion into the corresponding 5'-monophosphate derivative. Therefore it was concluded that 5-BV-Urd, and particularly 3'-AOD-5-MeUrd, are not significant substrates of TK-2 and should be considered as new and potent inhibitor lead compounds of the mitochondrial TK-2.

DISCUSSION

TK-2-mediated phosphorylation of pyrimidine nucleoside analogues may be important in G0/G1 cells since TK-2 is constitutively expressed throughout the cell cycle in contrast with the

cell cycle-regulated TK-1 that is predominantly expressed during the S-phase of the cell cycle [1]. Tissues affected by antiviral nucleoside toxicity such as muscle and liver, consist mainly of resting and terminally differentiated cells that often contain measurable levels of TK-2, but undetectable levels of TK-1. The mitochondrial location of TK-2 has been implicated as a determinant for mitochondrial DNA damage caused by several antiviral pyrimidine nucleoside analogues [16,17]. However, the importance of nucleoside analogue phosphorylation in the mitochondrial matrix for mitochondrial DNA damage is still unclear. For example, BVDU is known to be an efficient substrate for TK-2, yet has never been reported to cause mitochondrial toxicity in BVDU-treated patients [18–21]. On the other hand, ddC (Zalcitabine), known to cause mitochondrial toxicity and peripheral neuropathy in drug-treated patients [22,23], has a relatively poor affinity for TK-2 [9]. It has been suggested that ddCTP, formed in the cytosol or nucleus through the action of 2'-deoxycytidine kinase, is recognized by a transport carrier on the mitochondrial membrane and most probably imported into the mitochondria through this pathway to cause mitochondrial toxicity [24]. Thus the direct role of TK-2 in the mitochondrial toxicity of a number of drugs, including ddC, is still unclear. On the other hand, specific TK-2 inhibitors cannot be converted into a metabolite that directly causes damage to the mitochondrial DNA, and thus potential antimetabolic effects of such TK-2 inhibitors in the mitochondria should be unrelated to those found for nucleoside analogues that display an efficient TK-2 substrate activity and for which TK-2 acts as the activating enzyme. Therefore the use of efficient TK-2 inhibitors may not only resolve fundamental questions as to the role of TK-2 in pyrimidine nucleotide synthesis and the functioning and integrity of mitochondria (including mitochondrial DNA repair), but may also reveal and clarify the role of TK-2-catalysed phosphorylation (activation) in the mitochondrial toxicity of a variety of antiviral and anticancer drugs.

To date, no specific inhibitors of TK-2 have been described. In the present study we disclose two different lead compounds that proved quite selective in their inhibitory effect on mitochondrial TK-2, and that were able to inhibit TK-2 activity at concentrations that fell within the lower micromolar range. Surprisingly, both compounds are ribofuranosyl nucleoside analogues substituted either at the 5-position of the base moiety (5-BV-Urd) or at the 3'-position of the ribosyl moiety (3'-AOD-5-MeUrd). These findings were unexpected since it was assumed that TK-2 (just like TK-1) only recognized natural 2'-deoxyribonucleosides (i.e. dThd, dUrd and dCyd), unnatural 2'-deoxyribonucleoside analogues (i.e. BVDU) or arabinosyl nucleoside analogues (i.e. FIAU and araT) as efficient substrates. The present study reveals that TK-2 is also able to recognize ribofuranosyl nucleoside analogues with high affinity (K_i/K_m of 1–10), whereas cytosolic TK-1 did not exhibit any affinity at 500-fold higher compound concentrations. As far as we are aware, this is the first demonstration of a marked and specific recognition of ribonucleosides by a mammalian 2'-deoxynucleoside kinase. Although the affinity of 5-BV-Urd and 3'-AOD-5-MeUrd for TK-2 was quite pronounced, the catalytic (phosphorylating) efficacy of TK-2 for both compounds proved to be very low (5-BV-Urd) or even undetectable (3'-AOD-5-MeUrd) under our experimental conditions. These compounds should therefore be considered as efficient (competitive) inhibitors of TK-2 rather than substrates.

The novel inhibitors described in the present study have not been evaluated against native purified TK-2. However, earlier studies have shown that the substrate specificity of the recombinant enzyme is identical to that described for purified native

human TK-2 [4–6]. Also, the antiherpetic drug BVDU had comparable substrate properties against both native and recombinant TK-2 [9,15]. Thus we may expect that the inhibitory properties described in the present study for 5-BV-Urd and 3'-AOD-5-MeUrd against recombinant TK-2 may be comparable for native TK-2.

In the absence of a crystallographic X-ray structure of mitochondrial TK-2, it is impossible to speculate how the compounds fit into the enzyme and why their affinities for TK-2 are so high but their V_{max} values are so low compared with the natural substrates. It is currently unknown whether the compounds are able to penetrate the mitochondrial matrix to efficiently inhibit TK-2 activity *in situ*. Future studies should aim at clarifying this issue. However, we believe that the lead compounds that have emerged from our study will enable rational and targeted synthesis of more potent inhibitors of TK-2 that are active against the enzyme in the nanomolar range. Such novel inhibitors will be of great value to address the role of TK-2 in mitochondrial homeostasis (i.e. DNA repair) at concentrations that fully inhibit (knock-out) the catalytic activity of TK-2. However, they may also become of potential practical use when added at subtoxic concentrations (that partially suppress TK-2 activity) in combination with antiviral and anticancer agents that display toxic side-effects consequent to their activation by TK-2 in the mitochondrial environment.

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