Thymidine kinase and uridine-cytidine kinase from *Entamoeba histolytica*: cloning, characterization, and search for specific inhibitors

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SUMMARY

Entamoeba histolytica is an intestinal parasite and the causative agent of amoebiasis, which is a significant source of morbidity and mortality in developing countries. Although anti-amoebic drugs such as metronidazole, emetine, chloroquine and nitazoxanide are generally effective, there is always potential for development of drug resistance. In order to find novel targets to control *E. histolytica* proliferation we cloned, expressed and purified thymidine kinase (Eh-TK) and uridine-cytidine kinase (Eh-UCK) from *E. histolytica*. Eh-TK phosphorylates thymidine with a K_m of 0·27 μM, whereas Eh-UCK phosphorylates uridine and cytidine with K_m of 0·74 and 0·22 mM, respectively. For both enzymes, ATP acts as specific phosphate donor. In order to find alternative treatments of *E. histolytica* infection we tested numerous nucleoside analogues and related compounds as inhibitors and/or substrates of Eh-TK and Eh-UCK, and active compounds against *E. histolytica* in cell culture. Our results indicate that inhibitors or alternative substrates of the enzymes, although partially reducing protozoan proliferation, are reversible and not likely to become drugs against *E. histolytica* infections.

Key words: *Entamoeba*, amoebiasis, kinases, inhibitors, drugs.

INTRODUCTION

Entamoeba histolytica is an intestinal parasite and the causative agent of amoebiasis, which is a significant source of morbidity and mortality in developing countries (Stanley, 2003). The WHO estimates that *E. histolytica* causes severe disease in about 30–50 million people each year, killing 40 000–100 000 each year (WHO, 1997). The hyperendemic areas are located, in particular, in developing intertropical countries (Gatti *et al.* 2002), whereas in industrialized areas amoebic infections are rare. However, the constant increase in the number of immigrants from, and travellers to, endemic tropical areas accounts for a number of 'imported' cases of infection in developed countries. *E. histolytica* is also a highly pathogenic agent which could be used as a bioterror agent, a fact which has resulted in its designation as a NIAID Category B priority pathogen.

There have been few recent reports of development of novel anti-amoebic drugs (Seifert *et al.* 2001; Makioka *et al.* 2002; Ranque *et al.* 2004; Rossignol *et al.* 2007). So far 4 groups of anti-amoebic drugs are known: metronidazole, the major drug of choice and other nitroimidazole derivatives, such as diloxanide furoate; emetine; chloroquine (Bansal *et al.* 2004), and nitazoxanide. Active metabolites of metronidazole appear to bind to *E. histolytica* enzymes and diminish amoebic activity (Leitsch *et al.* 2007). Nitazoxanide is a thiazolide derivative with a wide spectrum of activity against anaerobic bacteria, parasites and viruses, and has the advantage that it is active against luminal parasites (Rossignol *et al.* 2007). Although these compounds effectively cure parasitic infections, it has been reported that metronidazole resistance can be induced in an axenic strain of *E. histolytica* following continuous exposure to steadily increasing drug concentrations (Samarawickrema *et al.* 1997), although Wassmann *et al.* (1999) reported only moderate resistance development in the laboratory. Thus, the potential for development of clinical drug resistance is always present (Bansal *et al.* 2006).

From the genome sequence now available (Loftus *et al.* 2005) it appears that, like other protozoa, *E. histolytica* does not possess enzymes for the *de novo* synthesis of nucleotides, thus relying on salvage pathways for the nucleotide supply needed for

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genome replication and RNA synthesis. We have hypothesized that enzymes involved in the salvage pathway of nucleotides could represent targets to control parasite proliferation in general (Maga *et al.* 1994; Strosselli *et al.* 1998). Two specific enzymes of the salvage pathway of nucleotides are encoded by the *E. histolytica* genome – thymidine kinase (Eh-TK) (EC 2.7.1.21) and uridine-cytidine kinase (Eh-UCK) (EC 2.7.1.48). Phylogenetic analysis suggests that Eh-TK clusters with eukaryotic TKs, not bacterial TKs such as has been found for *Cryptosporidium parvum* TK (Striepen *et al.* 2004). These enzymes are responsible for the first step of activation of thymidine, uridine and cytidine to their respective monophosphate forms, which are then converted to triphosphates by other cellular kinases. Specific inhibitors of these enzymes could prevent the production of key components of the parasitic nucleic acids. Furthermore, compounds which act as alternative substrates of these enzymes could be incorporated in growing nucleic acid chains and then block DNA replication or RNA transcription. In both cases this will result in the inhibition of the proliferation of the parasite.

MATERIALS AND METHODS

Preparation of E. histolytica *DNA*

E. histolytica DNA was extracted from 4 mg (wet weight) of protozoa by using the 'Wizard SV Genomic DNA Purification System' (Promega), following the instructions of the manufacturer.

Construction of recombinant bacterial expression vectors for H. histolytica *TK and UCK*

The complete genome of *E. histolytica* is available online at *Entamoeba histolytica Genome Project* (www.tigr.org/tdb/e2k1/ehe/). In order to amplify the coding sequences of Eh-TK (accession no: EHI_177540) and Eh-UCK (accession no: EHI_193300), 100 ng of purified *E. histolytica* DNA was PCR-amplified by using the following primers: for Eh-TK, 5′-GAAGCTAGCATGAATGAAAGTATAAGTG-3′ (sense) and 5′-AATGAGAATTCTTTCTTTTTTTTTCTTGAC-3′ (antisense); for Eh-UCK, 5′-CTTGCTAGCATGAATAGTACTGTAAG-3′ (sense) and 5′-TAACGAATTCTCTAATAAAATAAAATTTCAC-3′ (antisense), containing restriction sites for *NheI* in both sense primers and for *EcoRI* in both antisense primers. The amplified regions were inserted into the multiple cloning site of pTrcHisA plasmid (Invitrogen), also restricted with *NheI* and *EcoRI*, to give the recombinant bacterial expression vectors pTrcHis-EhTK and pTrcHis-EhUCK containing the complete sequences encoding for His-tagged proteins of 232 and 274 amino acids, respectively.

Expression and purification of recombinant Eh-TK and Eh-UCK from E. coli

Fifty μl of competent *E. coli* cells (SELECT 96™, Promega) were separately transformed by pTrcHis-EhTK and pTrcHis-EhUCK (1–50 ng) following the heat-shock protocol provided by the company, briefly 30 min on ice, 30 sec at 42 °C, 2 min on ice, then incubation in SOC medium (5 g/L bacto-yeast extract, 20 g/L bacto-tryptone, 0·5 g/L NaCl (Gibco), 2·5 mM KCl, 20 mM glucose) for 1 h at 37 °C. Expression and purification of the Eh-TK/Eh-UCK proteins were carried out as described by the manufacturer of the Ni-NTA Superflow resin (QIAGEN). Briefly, a fresh overnight saturated culture of *E. coli* transformed with the recombinant DNA was diluted 1 : 100 in 1 L of LB broth (5 g/L bacto-yeast extract, 10 g/L bacto-tryptone and 10 g/L NaCl) containing ampicillin (60 μg/ml), and incubated at 37 °C with shaking. At 0·6 OD_{600}, isopropylthio-β-D-galactoside (IPTG, Sigma) was added to a final concentration of 1 mM, and the culture was incubated for further 4 h at 37 °C. The bacterial cell pellet was resuspended in 4 volumes of lysis buffer (50 mM sodium phosphate, pH 8·0, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF and 1 mg/ml lysozyme) and incubated on ice for 30 min. Cells were then sonicated on ice, and the lysate was centrifuged at 10 000 ***g*** for 30 min at 4 °C. The supernatant was loaded on a Ni-NTA Superflow column (1 ml) at a flow rate of 0·25 ml/min. The Ni affinity chromatography was carried out under native conditions, and 10 column volumes of buffers were used in each step. The column was first washed with lysis buffer and then with 50 mM sodium phosphate buffer (pH 8·0), containing 300 mM NaCl and 20 mM imidazole. The protein was then step-eluted with 250 mM imidazole in 50 mM sodium phosphate buffer (pH 8·0) and 300 mM NaCl. Fractions were collected for enzymatic activity analysis. The enzymes, collected from peak fractions, were dialysed against 50 mM Tris-HCl (pH 7·5) and 1 mM DTT. The enzymes were rendered 20% glycerol and 0·5 mM PMSF, frozen in aliquots in liquid nitrogen and then stored at −80 °C until used.

Thymidine kinase assay

Recombinant Eh-TK, purified as above described, was assayed at 37 °C for 20 min in 15 μl of a mixture containing 30 mM K-phosphate, pH 8·0, 1·2 mM $MgCl_2$, 1 mM ATP, 0·5 mM DTT, and 0·5 μM [^{3}H-methyl]-Thd (1600 cpm/pmol, GE Healthcare). The reaction was terminated by spotting 10 μl of the incubation mixture on a 96-square DEAE sheet (DEAE Filtermat, Wallac). The DEAE sheet was washed 3 times in an excess of 1 mM ammonium formate, pH 5·3, in order to remove unconverted nucleoside, and finally in ethanol. The DEAE sheet was

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dried, covered by MeltiLex scintillator fluid, and radioactivity was counted in a PerkinElmer MicroBeta counter. One unit (U) is the amount of enzyme that phosphorylates 1 nmol of Thd to TMP in 1 h at 37 °C.

Uridine-cytidine kinase assay

Recombinant Eh-UCK, purified as above described, was assayed at 37 °C for 20 min in 15 μl of a mixture containing 50 mM Tris-HCl, pH 7·5, 2·4 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, and 0·8 mM [^{3}H]-Urd (8 cpm/pmol). The reaction was terminated by spotting 10 μl of the incubation mixture on a 96-square DEAE sheet (DEAE Filtermat, Wallac). Radioactivity was counted as described above. One unit (U) is the amount of enzyme that phosphorylates 1 nmol of Urd to UMP in 1 h at 37 °C.

Inhibition assays

Candidate inhibitors were available from previous work or purchased from commercial sources. Stock solutions of candidate inhibitors in DMSO were diluted into assay mixtures, and the latter were serially diluted to give at least 5 concentrations of test compound. Assays were carried out in duplicate as described above, in the presence of 0·27 μM [^{3}H]-Thd (TK) or 0·8 mM [^{3}H]-Urd (UCK). Each experiment was performed twice.

Phosphorylation assay

When nucleoside analogues were tested as possible substrates of *E. histolytica* enzymes, each compound (100 μM) was incubated at 37 °C for 30 min in the specific reaction mixture (25 μl) and with the required amount of enzyme. Samples were heated at 100 °C for 5 min and centrifuged for 15 min at 10 000 rpm in an Eppendorf bench-fuge. Supernatants were transferred to a new tube for subsequent HPLC analysis. The reverse phase chromatography method employed a HPLC system (SHIMADZU) to separate nucleosides from nucleotides. A Chrom-Sep C18 Column SS (4·6 × 150 mm; Varian) was used at room temperature under the following conditions: injection volume, 20 μl; detection, UV 260 nm; solvents of the linear gradient eluent: buffer A (20 mM KH_2PO_4, pH 7·5), buffer B (20 mM KH_2PO_4, pH 5·2, 60% methanol); linear gradient from 0% to 100% buffer B, over 40 min; flow rate, 0·5 ml/min.

Cell culture susceptibility assay

E. histolytica zymodeme II, isolated from a patient with acute intestinal infection, was used in cell culture tests. The strain was cultured in Robinson's monoxenic medium at 37 °C and subjected to electrophoretic analysis according to Sargeaunt's method (Sargeaunt *et al*. 1978). For drug sensitivity tests, trophozoites in exponential growth (24–48 h) were concentrated by centrifugation at 500 ***g*** for 10 min and counted in a haemocytometer. The parasite count was adjusted to 1×10^5/ml for experiments. Each experiment included metronidazole (MNZ) as a standard amoebicidal drug and control cultures without drug (culture medium with 1% DMSO).

The compounds tested were dissolved in DMSO, and diluted into assay medium to result in a final DMSO concentration of 1%. The assays were performed in 2·0 ml sterile cryovials, and serial 2-fold dilutions of the drugs were made in the liquid phase of Robinson's medium, in order to obtain a final volume of 1·5 ml of medium. Fifty μl of the trophozoite suspension (1×10^5 amoebae/ml) were added to each vial, and the vials were incubated at 37 °C. Each drug dilution was tested in triplicate, and the experiments were repeated 3 times. After 48 h the cultures were checked microscopically, and parasites counted in a haemocytometer chamber. The cultures were subcultured by inoculating 100 μl of drug-containing culture into fresh medium (1·5 ml). The subcultures were incubated at 37 °C, and parasites counted at 24 and 48 h in a haemocytometer chamber. In order to test parasite viability, the cultures were subcultured by inoculating 100 μl of drug-containing culture into fresh medium (1·5 ml) and incubating at 37 °C. At 24 and 48 h the amoebae were counted again. The minimum amoebicidal concentration (MAC) is defined as the lowest drug concentration that caused complete trophozoite destruction.

RESULTS AND DISCUSSION

Cloning, expression and purification of Eh-TK and Eh-UCK

The complete coding sequences of both putative enzymes were amplified by PCR and cloned in *E. coli* as described in the Materials and Methods section. Plasmids containing Eh-TK and Eh-UCK were sequenced in order to demonstrate the absence of mutations. When compared with *Homo sapiens* thymidine kinase 1 (TK1), Eh-TK exhibits 56% identity and 76% similarity at the amino acid (AA) sequence level, with 25 AA directly involved in thymidine and ATP interactions identical or similar in both enzymes (Fig. 1A).

From its sequence homology, Eh-UCK appears to belong to the nucleoside monophosphate (NMP) kinase fold family (Suzuki *et al*. 2004). Comparison of the Eh-UCK sequence against that of *Homo sapiens* UCK2, crystallized alone and in complexes with a substrate (Cyd), a feedback inhibitor (CTP or UTP), and with phosphorylation products (CMP or

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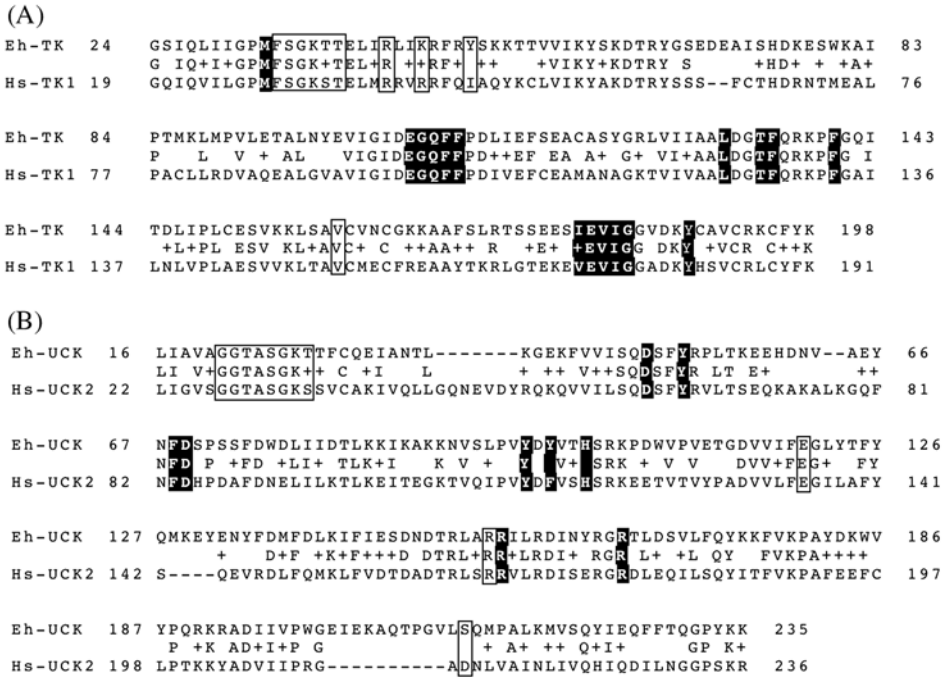

Fig. 1. Structural alignment of the sequences of Eh-TK and *Homo sapiens* TK1 (Hs-TK1) (panel A), and Eh-UCK and *Homo sapiens* UCK2 (Hs-UCK2) (panel B). Black boxes indicate the amino acids involved in interactions with the substrate (thymidine and cytidine for TK and UCK, respectively). Open boxes indicate the amino acids of TK and UCK involved in the interactions with the phosphate donor, ATP.

ADP), indicates 42% identity and 63% similarity at the AA level, and a strict homology in the conserved residues involved in catalysis and substrate binding (Fig. 1B).

Both enzymes were expressed in pTRC-HisA vector (Invitrogen) as N-terminal fusion proteins containing 6 tandem histidine residues that allow one-step purification with a nickel-chelating resin. One litre of bacterial culture produced approximately 10 mg of tagged Eh-TK and 2 mg of tagged Eh-UCK, both eluted from a Ni-NTA superflow column (1 ml) as single sharp peaks. The molecular masses of purified enzymes were 26 kDa and 40 kDa for Eh-TK and Eh-UCK, respectively (Fig. 2). Assayed under their optimal conditions the specific activities of Eh-TK and Eh-UCK were 1000 and 10 000 U/mg, respectively. Because of their high specific activity, both enzymes were diluted in 30 mM Hepes-K^+, pH 8·0, 20% glycerol and 1 mM DTT, before use.

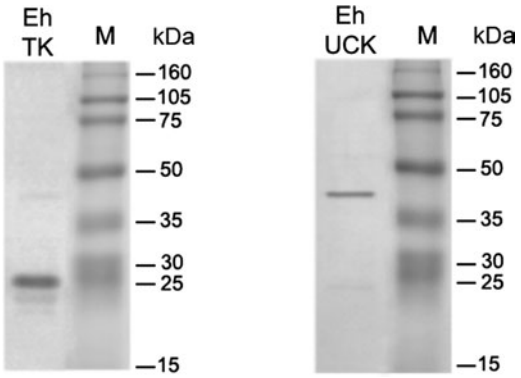

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electropherograms of the affinity-purified recombinant Eh-TK and Eh-UCK. Lanes: Eh-TK (3 μg) and Eh-UCK (1 μg) of recombinant enzyme eluted from the Ni-NTA column (90% pure by densitometry). M, molecular mass markers.

Biochemical characterization of Eh-TK and Eh-UCK

Once cloned, expressed and purified, both enzymatic activities were evaluated in different assay environments in order to determine their optimal assay conditions. As shown in Fig. 3, Eh-TK exhibits best activity at pH 8·0 at an ATP concentration range between 0·2 and 4·0 mM. $MgCl_2$ is required for Eh-TK activity, reaching its optimal effect at 1·2 mM when ATP is present at 1 mM. $MnCl_2$ can replace $MgCl_2$ in the assay, with optimal activity at 200 μM when ATP is present at 1 mM.

Eh-UCK shows its best activity at pH 7·6 with ATP at 2 mM. Eh-UCK also requires $MgCl_2$, reaching its optimal activity when present at 2·5 mM. Both Eh-TK and Eh-UCK are inhibited by KCl, with IC_{50} values of 160 and 250 mM, respectively (data not shown).

Eh-TK efficiently phosphorylates Thd, with a K_m value of 0·27 μM (Table 1) when ATP is phosphate donor. The other 3 deoxyribonucleosides and the

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Table 1. Kinetic properties of recombinant Eh-TK and Eh-UCK with ATP as phosphate donor[a]

Eh-TK:

Substrate	K_m (μM) ± S.D.	V_{max} (pmol min^{-1} μg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Thd	0·27 ± 0·03	206 ± 10	9×10^{-2}	$3{\cdot}3 \times 10^{5}$

Eh-UCK:

Substrate	K_m (mM)	V_{max} (pmol min^{-1} μg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Urd	0·74 ± 0·10	2200 ± 200	1·17	$1{\cdot}6 \times 10^{3}$
Cyd	0·22 ± 0·01	851 ± 100	$4{\cdot}5 \times 10^{-1}$	$2{\cdot}1 \times 10^{3}$

[a] Kinetic experiments were done twice in triplicate, and parameters were obtained using Prism 3 for MAC (version 3.0cx).

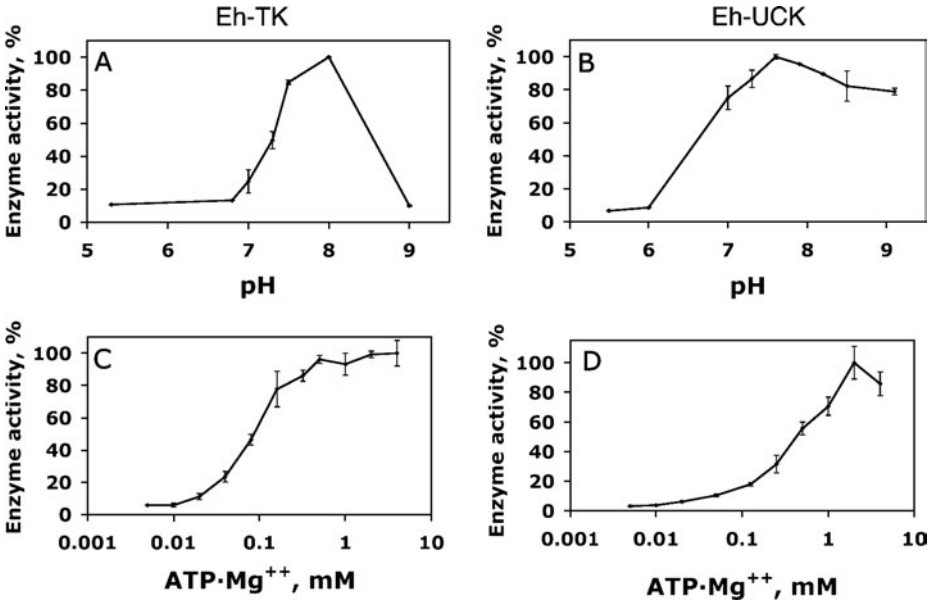

Fig. 3. Effect of pH (K-phosphate buffer, 30 mM) and ATP•Mg^{++} concentration on activity of Eh-TK (panels A and C) and Eh-UCK (panels B and D).

4 ribonucleosides are not substrates of Eh-TK, nor do NTPs other than ATP support activity (data not shown). Eh-UCK phosphorylates Urd and Cyd with K_m values of 0·74 and 0·22 mM, respectively (Table 1). Eh-UCK catalyses phosphate transfer preferentially from ATP. GTP replaces ATP but with only about 25% of the efficiency of the latter (data not shown), while UTP and CTP do not replace ATP. The fact that the substrate Cyd is a competitive inhibitor of the phosphorylation of [^{3}H]-Urd by Eh-UCK suggests that Cyd and Urd share the same active site (data not shown).

Search for specific inhibitors

In order to find specific, non-substrate inhibitors of the salvage pathway of pyrimidine nucleosides or alternative substrates which could interfere with DNA synthesis, we screened approximately 160 nucleoside analogues and related compounds for their ability to inhibit the phosphorylation of Thd and Urd catalysed by Eh-TK and UCK, respectively, as described in the Materials and Methods section. The names and IC_{50} values of the active compounds are reported in Table 2.

5-Trifluoromethyl-2′-deoxyuridine (TFT), 5-iodo-2′-deoxyuridine (IdU), β-5-ethyl-2′-deoxyuridine (EdU) and β-L-thymidine (β-L-T) were potent inhibitors of the catalytic activity of Eh-TK, with IC_{50} values of 0·6, 1, 2·5 and 5 μM, respectively (Table 2). In contrast to β-L-T (see below), TFT, IdU and EdU were efficiently phosphorylated by Eh-TK (data not shown), confirming that these compounds enter the active site of the enzyme and act as alternate substrates. Thus, they could potentially interfere with protozoal DNA synthesis if they are further phosphorylated to the triphosphates by nucleotide kinases.

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Table 2. Effects of nucleoside analogues on Eh-TK activity (IC_{50})[a]

Nucleoside analogue	Acronym	IC_{50} (μM) ± S.D.[b]
5-Trifluoromethyl-2′-deoxyuridine	TFT	0·6 ± 0·1
5-Iodo-2′-deoxyuridine	IdU	1 ± 0·25
β-L-Thymidine	β-L-T	5 ± 1
α-L-Thymidine	α-L-T	35 ± 10
α-D-5-Ethyl-2′-deoxyuridine	α-EdU	17 ± 3·4
β-D-5-Ethyl-2′-deoxyuridine	EdU	2·5 ± 0·5
Penciclovir	PCV	10 ± 2·2
6-Methyluridine	6-MeU	15 ± 4·2
3′-Ethyl-5-methyl-2′-deoxycitidine	EMC	18 ± 3·9
D-5-(Bromovinyl)-2′-deoxyuridine	BvdU	> 100 ± 20
L-5-(Bromovinyl)-2′-deoxyuridine	L-BvdU	> 100 ± 20
5-Propyl-2′-deoxyuridine	PdU	44·4 ± 11
2-Phenylamino-9-(4-hydroxybutyl)-6-oxopurine	HBPG	> 100 ± 20

[a] Enzyme assays contained 0·27 μM [^{3}H]-Thd and were run in duplicate with at least 5 concentrations of inhibitor.
[b] The values are the means for 2 independent experiments in which each concentration was tested in duplicate.

Table 3. Effects of nucleoside analogues on Eh-UCK activity (IC_{50})[a]

Nucleoside analogue	Acronym	IC_{50} (μM) ± S.D.[b]
6-(4-Hexyloxyanilino)uracil	HexO-AU	54 ± 12
5-Propyl-2′-deoxyuridine	PdU	90 ± 21

[a] Enzyme assays contained 0·8 mM [^{3}H]-Urd and were run in duplicate with at least 5 concentrations of inhibitor.
[b] The values are the means for 2 independent experiments in which each concentration was tested in duplicate.

The strongest inhibitors of Eh-UCK were 5-propyl-2′-deoxyuridine (PdU) and a uracil base analogue 6-(4-hexyloxyanilino)uracil (HexO-AU) with IC_{50} values of 90 and 54 μM, respectively (Table 3). These compounds were inactive against human UCK1, previously cloned and purified (Roy *et al.* 2004) (data not shown). The fact that out of 160 different nucleoside analogues tested against Eh-UCK (data not shown), only PdU and HexO-AU showed significant inhibition suggests that, in contrast to Eh-TK, Eh-UCK is highly specific for its natural substrates. Indeed, by studying the kinetics of phosphorylation of Urd by Eh-UCK at different concentrations of substrate or phosphate donor, it appears (Fig. 4) that PdU competes with ATP but not with Urd.

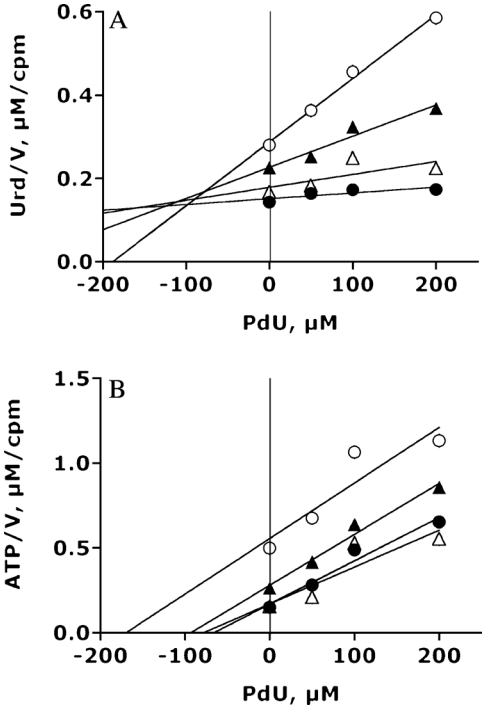

Fig. 4. Hanes-Woolf plot showing the effect of increasing concentrations of PdU on Eh-UCK activity in the presence of different concentrations of Urd (panel A) and ATP (panel B). Panel A: [^{3}H]-Urd at 0·25 (●), 0·5 (△), 1 (▲) and 2 mM (○). Panel B: ATP at 0·2 (●), 0·4 (△), 0·8 (▲) and 1·6 mM (○) in the presence of 0·8 mM [^{3}H]-Urd.

Eh-TK is enantioselective

In this work we also found that L-thymidine (β-L-T), although an inhibitor of Eh-TK, is poorly phosphorylated by the enzyme. When saturating amounts of Eh-TK were incubated in conditions leading to complete conversion to the 5′-monophosphate of the natural substrate Thd, only weak phosphorylation of β-L-T was observed (<5% compared with the natural substrate). This is consistent with the observed non-competitive inhibition of β-L-T *vs* the natural substrate Thd (data not shown). Thus, Eh-TK shows catalytic behaviour different from both human and herpes simplex virus TKs, where human TK1 is strictly enantioselective and herpesvirus TKs are not enantioselective (Spadari *et al.* 1992, 1998). Furthermore, like herpes simplex virus TKs, Eh-TK is strongly inhibited by

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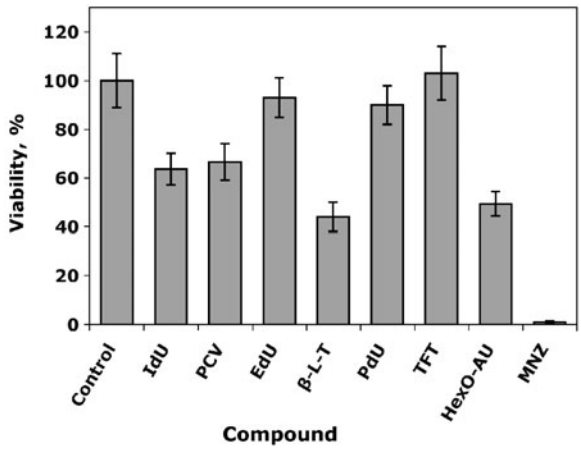

Fig. 5. Effect of Eh-TK and Eh-UCK inhibitors on *Entamoeba histolytica* proliferation showing the relative percentage of cells present in cell culture after 48 h in the presence of 200 μM of the compounds. All cultures contained 1% DMSO. Bars are the means ± S.D. of 3 independent experiments.

D-5-iododeoxyuridine (IdU) but, in contrast to the herpes enzymes, it is not inhibited by the corresponding L-enantiomer (data not shown).

Effect of Eh-TK and Eh-UCK inhibitors on E. histolytica *proliferation in cell culture*

The interesting enzyme inhibition results prompted testing of the most active compounds against *E. histolytica* proliferation in cell culture. We decided to carry out the experiments with a 'wild type' strain rather than a reference strain, e.g. HM-1:IMSS. The latter strain is forced to live in artificial conditions and may contain mutations that modify its virulence. *E. histolytica* cultures were exposed to 200 μM of the compounds for 48 h, and then subcultured in drug-free medium for an additional 48 h (see Materials and Methods section). Compared with the potent effect of metronidazole (MNZ), all tested compounds showed weak inhibitory activity after 48 h of exposure (Fig. 5). The cultured trophozoites appeared rounded with many intracytoplasmic vacuoles, but remained viable. However, after the removal of the test compounds *E. histolytica* cultures completely recovered their growth. These results indicate a low degree of susceptibility of the parasite to these compounds, suggesting that none of them warrants further development to control *E. histolytica* proliferation.

The lack of cytotoxicity of the compounds found active *in vitro* could be due either to an efficient mechanism of degradation or excretion of the drugs, or to the presence of high levels of nucleoside monophosphates in the cytoplasm of bacteria phagocytosed from the medium. In particular, TFT, IdU and EdU, which are activated to 5′-monophosphates by Eh-TK, are probably incorporated into protozoal DNA by DNA polymerases and can be responsible for their cytostatic effect. The weak cytotoxic effect of these compounds against protozoal proliferation could be due either to the fact that they are poor substrates of *E. histolytica* DNA polymerases or to efficient DNA repair mechanisms.

On the other hand, it is not yet possible to rule out the presence in the parasite of another, undescribed *de novo* pathway which could overcome the block of the salvage pathway. The *E. histolytica* genome contains additional putative TK and UCK genes. Besides the TK cloned here (XP 655924), there is a second TK with very little sequence similarity that may be a bacterial enzyme. In addition to the UCK cloned here (XP 651955), the genome contains 4 more members (XP 648919, 656795, 651360 and 651299). XP 651299 is similar to the characterized sequence although it is carboxy-terminal extended, and XP 648919 is of similar size and about 50% sequence identity (Loftus *et al*. 2005).

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