Cloning and characterization of thymidine kinase and uridine-cytidine kinase from Entamoeba histolytica. Search for specific inhibitors

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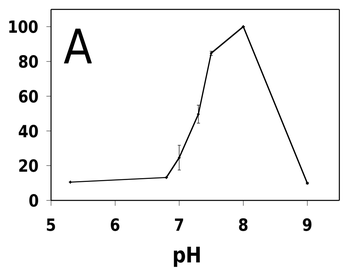
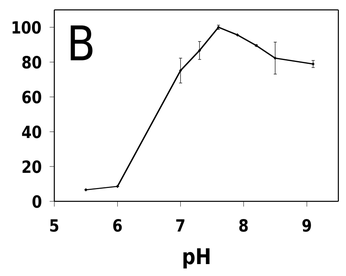
INTRODUCTION

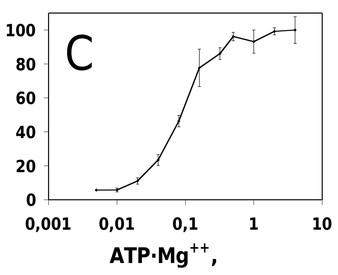
Entamoeba histolytica is an intestinal parasite and the causative agent of amoebiasis, which is a significant source of morbididy and mortality in developing countries. Antiamoebic drugs include metronidazole, the major drug of choice, and other nitroimidazole, emetine and chloroquine. Since the potential for development of drug resistance is always present and vaccine development appears to be a distant goal, we searched for novel possible targets for anti-Entamoeba chemotherapy.

Because *E. histolytica* relies for its supply of nucleotides only via the salvage pathway, we were confident that enzymes involved in this pathway could represent possible targets to control parasite proliferation. Based upon the genome sequence now available, we cloned, expressed and purified thymidine kinase (Eh-TK) and uridine-cytidine kinase (Eh-UCK) from *E. histolytica*.

Biochemical characterization of Eh-TK and Eh-UCK







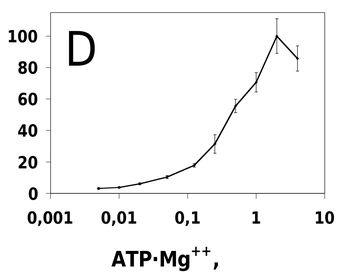


Fig. 1. Effect of pH (K-phosphate buffer, 30 mM) and $ATPMg^{++}$ concentration on Eh-TK (panel A and C) and Eh-UCK (panels B and D) activity.

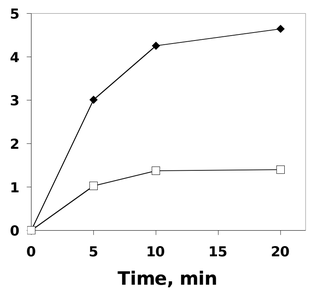


Fig. 2. Eh-UCK activity in presence of different phosphate donors (2 mM): ATP ( ), GTP ( ). Eh-TK use only ATP as phosphate donor.

Table I. Kinetic properties of recombinant Eh-TK and Eh-UCK with ATP as phosphate donor.

Eh-TK				
Substrate	K_m (μM)	V_{max} (pmol min^{-1} μg^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} M^{-1})
Thd	0.27 ± 0.03	16.8 ± 0.5	7.3×10^{-3}	2.7×10^{4}
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	883 ± 10	4.7×10^{-1}	6.3×10^{2}
Cyd	0.22 ± 0.01	340.6 ± 5.8	1.82×10^{-1}	8.26×10^{2}

Sequence alignment

(A)

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Eh-TK   24   GSIQLIIGPMFSGKTTELIRLIKRFRYSKKTTVVIKYSKDTRYGSEDEAISHDKESWKAI   83
             G IQ+I+GPMFSGK+TEL+R ++RF+ ++   +VIKY+KDTRY S     +HD+ + +A+
Hs-TK1  19   GQIQVILGPMFSGKSTELMRRVRRFQIAQYKCLVIKYAKDTRYSSS--FCTHDRNTMEAL   76

Eh-TK   84   PTMKLMPVLETALNYEVIGIDEGQFFPDLIEFSEACASYGRLVIIAALDGTFQRKPFGQI   143
             P   L  V + AL   VIGIDEGQFFPD++EF EA A+ G+ VI+AALDGTFQRKPFG I
Hs-TK1  77   PACLLRDVAQEALGVAVIGIDEGQFFPDIVEFCEAMANAGKTVIVAALDGTFQRKPFGAI   136

Eh-TK   144  TDLIPLCESVKKLSAVCVNCGKKAAFSLRTSSEESHIEVIGGVDKYCAVCRKCFYK   198
              +L+PL ESV KL+AVC+ C ++AA++ R  +E+ +EVIGG DKY +VCR C++K
Hs-TK1  137  LNLVPLAESVVKLTAVCMECFREAAYTKRLGTEKEVEVIGGADKYHSVCRLCYFK   191
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(B)

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Eh-UCK  16   LIAVAGGTASGKTTFCQEIANTL-------KGEKFVVISQDSFYRPLTKEEHDNV--AEY   66
             LI V+GGTASGK++ C +I   L        + ++ V++SQDSFYR LT E+        ++
Hs-UCK2 22   LIGVSGGTASGKSSVCAKIVQLLGQNEVDYRQKQVVILSQDSFYRVLTSEQKAKALKGQF   81

Eh-UCK  67   NFDSPSSFDWDLIIDTLKKIKAKKNVSLPVYDYVTHSRKPDWVPVETGDVVIFEGLYTFY   126
             NFD P +FD +LI+ TLK+I   K V +   Y  V+  SRK + V V   DVV+FEG+  FY
Hs-UCK2 82   NFDHPDAFDNELILKTLKEITEGKTVQIPVYDFVSHSRKEETVTVYPADVVLFEGILAFY   141

Eh-UCK  127  QMKEYENYFDMFDLKIFIESDNDTRLARRILRDINYRGRTLDSVLFQYKKFVKPAYDKWV   186
                  +   D+F +K+F+++D DTRL+RR+LRDI+ RGR L+ +L QY  FVKPA++++
Hs-UCK2 142  S----QEVRDLFQMKLFVDTDADTRLSRRVLRDISERGRDLEQILSQYITFVKPAFEEFC   197

Eh-UCK  187  YPQRKRADIIVPWGEIEKAQTPGVLSQMPALKMVSQYIEQFFTQGPYKK   235
              P +K AD+I+P G             + A+ ++ Q+I+     GP K+
Hs-UCK2 198  LPTKKYADVIIPRG----------ADNLVAINLIVQHIQDILNGGPSKR   236
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Fig. 3. Structural alignment of the amino acid sequences of Eh-TK and Hs-TK (panel A) with 56% identity and 76% similarity, and Eh-UCK and Hs-UCK2 (panel B)with 42% identity and 63% similarity. Black boxes indicate the aminoacids involved in the interactions with the substrate (thymidine and cytidine for TK and UCK, respectively). Open boxes indicate the aminoacids of TK and UCK involved in the interactions with the phosphate donor, ATP.

Search for inhibitors

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

Nucleoside analog	*Acronym*	*IC_{50} (μM) (mean ± SD)*[b]
5-Trifluoromethyl-2'-deoxyuridine	TFT	0.6 ± 0.1
5-Iodo-2'-deoxyuridine	IdUrd	1 ± 0.25
β-L-Thymidine	β-L-Thd	5 ± 1
α -L-Thymidine	α-L-Thd	35 ± 10
α-D-5-Ethyl-2Õdeoxyuridine	α-EdU	17 ± 3.4
β-D-5-Ethyl-2Õdeoxyuridine	β-EdU	2.5 ± 0.5
Penciclovir	PCV	10 ± 2.2
6-Methyluridine	6-MeU	15 ± 4.2
3ÕEthyl-5-methyl-2'-deoxycitidine	EMC	18 ± 3.9
D-5-(Bromovinyl)-2'-deoxyuridine	D-BVdU	>100 ±20
L-5-(Bromovinyl)-2'-deoxyuridine	L-BVdU	>100 ± 20
5-Propyl-2Õdeoxyuridine	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 five concentrations of inhibitor. [b] The values are the means for two independent experiments in which each concentration was tested in duplicate.

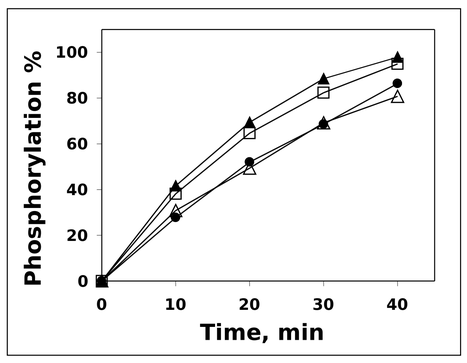
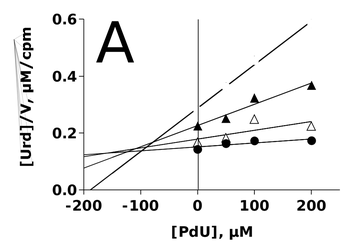


Fig. 4. Kinetics of phosphorylation of D-Thd ( ), TFT ( ), IdU ( ) and β-EdU ( ) by Eh-TK.

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

Nucleoside analog	*Acronym*	*IC_{50} (μM) (mean ± SD)*[b]
6-(4-hexyloxyanilino)uracil	HexO-AU	54 ± 12
5-Propyl-2Õdeoxyuridine	PdU	90 ± 21

[a] Enzyme assays contained 0.8 mM [^{3}H]-Urd and were run in duplicate with at least five concentrations of inhibitor. [b] The values are the means for two independent experiments in which each concentration was tested in duplicate.



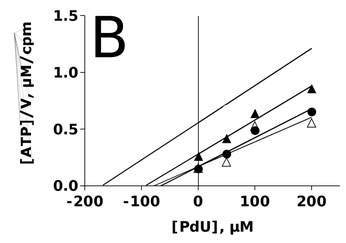


Fig. 5. Hanes-Woolf plot showing the effect of increasing concentrations of β-PdU on Eh-UCK activity in presence of different concentrations of Urd (panel A) and ATP (panel B). Panel A: concentrations of [^{3}H]-Urd: 0.25 mM ( ), 0.5 mM ( ), 1 mM ( ) and 2 mM ( ). Panel B: concentrations of ATP: 0.2 mM ( ), 0.4 mM ( ), 0.8 mM ( ) and 1.6 mM ( ) in the presence of 0.8 mM [^{3}H]-Urd.

Eh-TK is enantioselective

In this work we also demonstrate that L-Thd, although an inhibitor of Eh-TK, is not phosphorylated by the enzyme. In fact when saturating amounts of Eh-TK were incubated in conditions leading to the complete formation of the 5'-monophosphate of the natural substrate (D-)Thd, only weak phosphorylation of L-Thd was observed (<5% compared with the natural substrate). This is consistent with the observed non-competitive inhibition of L-Thd *versus* the natural substrate Thd (data not shown). Thus, Eh-TK shows catalytic behavior different from both human and herpes simplex virus TKs, where Hs-TK1 is strictly enantioselective and herpesvirus TKs are not enantioselective at all. Furthermore, like HSV TKs, Eh-TK is strongly inhibited by D-5-iododeoxyuridine (D-IdU) but, contrary to HSV TKs, it is not inhibited by the corresponding L-enantiomer (data not shown).

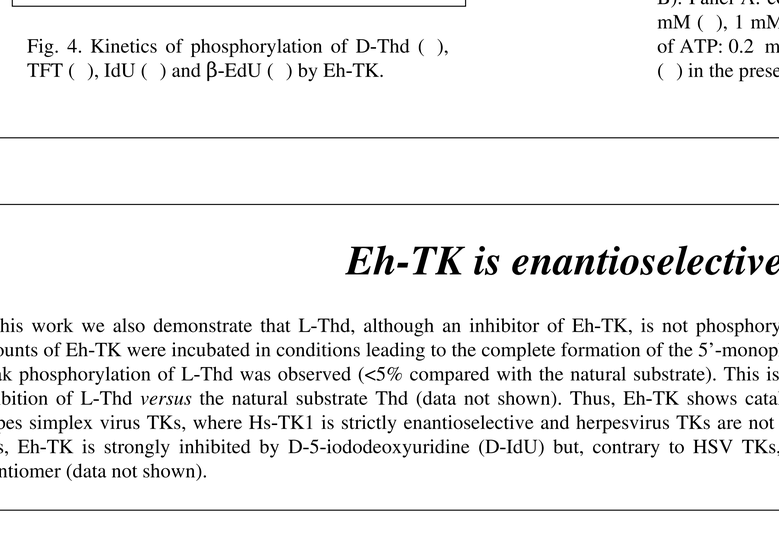


Fig. 6. Effect of Eh-TK and Eh-UCK inhibitors on *E. histolytica* proliferation showing the relative percentage of cells present in cell culture after 48 h in the presence of 200 μM (1% DMSO) of the compounds. Bars are the means ± SD of 3 independent experiments.

CONCLUSIONS

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 several nucleoside analogs, both *in vitro*, against Eh-TK and Eh-UCK, and in cell culture. Our results indicate that inhibitors or alternative substrates tested against both enzymes, althought partially reducing protozoan proliferation, are reversible and not likely to become drugs against *E. histolytica* infections.