Trichomonas vaginalis thymidine kinase: purification, characterization and search for inhibitors

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We report that a thymidine kinase (TK) activity is present in *Trichomonas vaginalis* and can be separated from the deoxyribonucleoside phosphotransferase. *T. vaginalis* TK, purified 11 200-fold to apparent homogeneity, has a molecular mass of 31 500 Da. It phosphorylates not only thymidine (K_m 0.18 μ M) but also deoxycytidine (K_m 0.88 μ M) and deoxyuridine (K_m 0.14 μ M). In contrast with *T. vaginalis* deoxyribonucleoside phosphotransferase, the TK activity is strongly inhibited by novel deoxyuridine analogues such as 5-methyl-4'-thio-2'-deoxy-

uridine (MTdU) (K_i 20 nM) and 5-iodo-4'-thio-2'-deoxyuridine (ITdU) (K_i 24 nM). MTdU and ITdU are phosphorylated by *T. vaginalis* TK *in vitro*. *In vivo* they inhibit [³H]thymidine incorporation in *T. vaginalis* cultured cells and *T. vaginalis* growth (IC_{50} 7.5 and 24 μ M respectively; minimal lethal dose 100 μ M). Thus the TK inhibitors described here demonstrate the key role of *T. vaginalis* TK for protozoal growth and viability and indicate TK as a new target for the design of antitrichomonal drugs.

INTRODUCTION

Trichomonas vaginalis, a protozoal parasite infecting humans, adheres to the epithelial layer of the infected urogenital tract and causes trichomonas vaginitis. Its inability to incorporate glycine, formate and bicarbonate into purines [1], as well as aspartate, orotate and bicarbonate into pyrimidines [2], indicates that *T. vaginalis* lacks purine and pyrimidine *de novo* biosyntheses. Therefore the parasite acquires the precursors of its nucleic acids by taking up DNA and RNA of lysed host cells and digesting them by its nucleases and phosphatases. Bases are then converted to nucleosides by the nucleoside phosphorylases; however, it remains controversial whether nucleosides are phosphorylated by kinases or phosphotransferases [1–3]. On the specific metabolism of thymidine in *T. vaginalis*, Wang and Cheng [2] reported that thymidine is converted to TMP only by a deoxyribonucleoside phosphotransferase (NP) that phosphorylates thymidine, deoxycytidine, deoxyadenosine and deoxyguanosine and therefore seems to be essential for supplying the parasite with the corresponding deoxyribonucleoside monophosphates. In contrast, Miller and Linstead [3] did not find any NP activity in *T. vaginalis* preparations but they observed that both ribo- and deoxyribopurine and pyrimidine nucleosides are phosphorylated in the presence of ATP [3], indicating the unique presence of kinase activities in *T. vaginalis*. Because differences in nucleotide metabolism were also observed between *T. vaginalis* and the related parasite *Tritrichomonas foetus*, which is also deficient in the synthesis *de novo* of purine [4] and pyrimidine [5] nucleotides, the discrepancy between Wang's and Miller's data on the presence or absence of kinase activities in *T. vaginalis* has been ascribed by Wang to possible differences between *T. vaginalis* strains [2].

In the present study we reinvestigated the enzymic activities

responsible for the provision of dTMP to the parasite and found both deoxyribophosphotransferase and thymidine kinase (TK) to be present in a *T. vaginalis* strain isolated from a patient. In particular, we purified TK and studied the effect of novel nucleoside analogues that can inhibit protozoan proliferation.

EXPERIMENTAL

Materials

Analytical grade reagents and solvents were used. [³H]Deoxythymidine ([³H]dT, 25 Ci/mmol) and [³H]deoxycytidine ([³H]dC, 18–30 Ci/mmol) were obtained from Amersham. [³H]Deoxyuridine ([³H]dU, 15.2 Ci/mmol) was from Moravsek Biochemicals. DE-81 filters were from Whatman. HiTrap-Q column and activated CH-Sepharose 4B were purchased from Pharmacia Fine Chemicals. PMSF and *p*-nitrophenylthymidine 3'-phosphate were from Sigma. Ultrapure SDS, acrylamide, bisacrylamide, ammonium persulphate and Bio-Rad protein assay were obtained from Bio-Rad.

Chemistry

5-Methyl-4'-thio-2'-deoxyuridine (MTdU) and 5-iodo-4'-thio-2'-deoxyuridine (ITdU) were synthesized as described by Dyson et al. [6] and Rahim et al. [7] respectively.

Preparation of *p*-aminophenylthymidine 3'-phosphate-CH-Sepharose affinity column

The procedure used was that described by Kowal and Markus [8] with the modifications by Tamiya et al. [9], which reduced *p*-nitrophenylthymidine 3'-phosphate to *p*-aminophenylthymidine-3'-phosphate. The reduced compound was coupled to activated

Abbreviations used: DTT, dithiothreitol; IdU, 5-iodo-2'-deoxyuridine; ITdU, 5-iodo-4'-thio-2'-deoxyuridine; MTdU, 5-methyl-4'-thio-2'-deoxyuridine; NP, deoxyribonucleoside phosphotransferase; TK, thymidine kinase.

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CH-Sepharose 4B by the method recommended by Pharmacia Fine Chemicals.

T. vaginalis cell culture

T. vaginalis, isolated from patients, was cultivated at 37 °C in CPLM (cysteine/peptone/liver/maltose) medium, pH 6.3, prepared as described by Diamond [10] without agar and with 10% (v/v) horse serum (Sigma). Cultures inoculated with 10% of a stationary-phase *T. vaginalis* cell culture (2×10^8 cells/ml) grew consistently to the mid-exponential phase of 6×10^5 cells/ml after a 48 h incubation. These mid-exponential-phase cells were used in all the subsequent studies.

Purification of *T. vaginalis* TK

T. vaginalis (9 g, mycoplasma-free) cells were resuspended in 45 ml of 20 mM Tris/HCl (pH 7.5)/1 mM dithiothreitol (DTT)/1 mM PMSF (buffer A). After 15 min on ice, cells were homogenized with a Dounce homogenizer (pestle A) and centrifuged at 10000 *g* for 15 min. The supernatant was collected and saved on ice. The pellet was resuspended in 10 ml of buffer A containing 0.2% (v/v) NP40, sonicated three times for 5 s at 50 W and centrifuged again as above. The combined supernatants (crude extract fraction) were centrifuged for 1 h at 100000 *g*. The supernatant (S-100 fraction) was loaded on a DEAE-Sepharose column (15 ml) at a flow rate of 1 ml/min. Enzyme was eluted by a linear gradient, from 0 to 1 M KCl in buffer A, as a single peak at 0.2 M KCl. The peak fractions (15 ml) were loaded on *p*-aminophenylthymidine 3'-phosphate-CH-Sepharose column (1 ml) equilibrated with 20 mM of Tris/HCl (pH 7.5)/1 mM DTT. The column was washed with the same buffer and the enzyme was eluted with 0.5 M Tris/HCl (pH 7.5)/200 μ M thymidine/1 mM DTT/0.3 mg/ml BSA. Fractions containing the TK activity were pooled then filtered on a NAP-5 column (Pharmacia) to remove thymidine; the enzyme was then frozen in liquid nitrogen until use. The final preparation had a specific activity of 11200 units/mg.

Purification of human TK

Human TK was purified with the *p*-aminophenylthymidine 3'-phosphate-CH-Sepharose affinity column as described by Focher et al. [11].

TK assays

TK activity was assayed at 37 °C for 30 min in 25 μ l of a mixture containing 30 mM Hepes/K⁺, pH 7.5, 6 mM MgCl₂, 6 mM ATP, 0.5 mM DTT and 1.6 μ M [³H]dT (2200 c.p.m./pmol). The deoxycytidine kinase and deoxyuridine kinase activities of *T. vaginalis* TK were determined in the presence of various concentrations of [³H]dC (2200 c.p.m./pmol) or [³H]dU (1330 c.p.m./pmol) in place of [³H]dT. Under these assay conditions a concentration of enzyme was used that would allow linear rates of phosphorylation of each substrate at all substrate concentrations. The reaction was terminated by spotting 20 μ l of the incubation mixture on a 25 mm DE-81 paper disk (Whatman). The disk was washed twice in an excess of 1 M ammonium formate, pH 3.6, to remove unconverted nucleoside, then twice in ethanol and finally it was dried. Radioactive dTMP was estimated by adding to the filter 1 ml of Betamax scintillating fluid (ICN) and counting in a β -counter. At low substrate concentrations, to increase the sensitivity of the assay approx. 6–8-fold, dried filters were placed in 20 ml scintillation vials, making sure they were flat on the vial bottom. 1 M KCl/0.1 M HCl solution (1 ml) was added and

radioactivity was extracted by shaking for 20–30 min. To each vial 4 ml of EcoLume (ICN) was added and the radioactivity was counted in a β -counter. One unit of TK is defined as the amount of enzyme that converts, under the above assay conditions, 1 nmol of thymidine into dTMP in 1 h.

When [γ -³²P]ATP was used in the TK assay, the enzyme was incubated in the mixture described above containing 1 mM [γ -³²P]ATP (125 c.p.m./pmol), 2 mM MgCl₂ and the compound under test (40 μ M). After 1 h at 37 °C the reaction was stopped by the addition of 1.5 μ l of 0.5 M EDTA. Each sample was then added to 2 μ l of a solution containing MTdU, ITdU, dT and dTMP (each at 10 mM) as optical markers. Samples were heated for 5 min at 80 °C and centrifuged at 10000 rev./min (8000 *g*) for 10 min. A 20 μ l sample of the supernatant was injected into the HPLC column.

NP assay

NP was assayed at 37 °C for 30 min in 25 μ l of a mixture containing 100 mM Hepes/K⁺, pH 6.8, 8 mM MgCl₂, 40 mM dUMP, 0.5 mM DTT and 20 μ M [³H]dT (440 c.p.m./pmol) or 20 μ M [³H]dC (440 c.p.m./pmol). The reaction was terminated by spotting 20 μ l of the incubation mixture on a 25 mm DE-81 paper disk. The disk was then washed as described above; radioactive dTMP or dCMP was estimated by adding to the filter 1 ml of Betamax scintillating fluid. Radioactivity was then counted in a β -counter. One unit of NP is defined as the amount of enzyme that converts, under the above assay conditions, 1 nmol of dT to TMP in 1 h.

Nucleoside and nucleotide separation by reverse-phase HPLC

The reverse-phase method employing the Bio-Rad 100 MAPS preparative system was used to separate nucleosides from nucleotides. A reverse-phase C₁₈ BioSil ODS-5S column (0.4 cm $\times$ 15 cm) was used at room temperature under the following conditions: injection volume, 40 μ l; detection, UV 260 nm; eluents, buffer A (20 mM KH₂PO₄, pH 5.6), buffer B [20 mM KH₂PO₄ (pH 5.6)/60% (v/v) methanol]. The gradient conditions were: 0–10 min, 0% buffer B; 10–16 min, 0–100% buffer B; 16–32 min, 100% buffer B. The flow rate was 0.5 ml/min. Altogether 32 fractions (fraction volume 0.5 ml) were collected and counted in a β -counter.

Thymidylate kinase assay

TK was incubated at 37 °C for 60 min in 15 μ l of a mixture containing 30 mM Hepes/K⁺, pH 7.5, 6 mM MgCl₂, 6 mM ATP, 0.5 mM DTT and 50 μ M [¹⁴C]TMP (1000 c.p.m./pmol). The reaction was terminated by spotting 3 μ l of the incubation mixture on a poly(ethyleneimine)-cellulose TLC sheet (Merck). The sheet was developed in 0.5 M ammonium formate, pH 3.6, and the positions of the labelled dTMP and dTDP were revealed by autoradiography. The sheet was cut and the amount of radioactive dTDP formed was estimated by liquid-scintillation counting in 1 ml of Betamax.

Determination of kinetic parameters (K_m and V_{max})

Analysis of substrate kinetics was done with the Michaelis-Menten equation in the form: $v = V_{max} - K_m(v/[S])$. Values of K_m were calculated by non-linear regression analysis with the Enzyme Kinetics program (performed by G. B. Gilbert, Indiana University, Bloomington, IN, U.S.A.). K_i values were determined by using the equation $K_i = [I]/([K_p/K_m] - 1)$, where $[I]$ is the concentration of the inhibitor and K_p is written for $K_m(1 + [I]/K_i)$.

Determination of $s_{20,w}$ by glycerol density ultracentrifugation

The sedimentation behaviour of *T. vaginalis* TK was determined by loading 100 μ l of enzyme preparation on 5 ml linear glycerol gradients [5–25 % (v/v)] in 20 mM phosphate buffer (pH 7.5)/1 mM DTT/10 % sucrose/0.1 mM EDTA.

After centrifugation in a Beckman L5-50 centrifuge in an SW50 rotor at 42000 rev./min (150000 *g*) for 26 h at 4 °C, 31 fractions, each 166 μ l, were collected from the tube and assayed for TK activity as described above. The following markers were run in the parallel tubes (given as $s_{20,w}$ values): γ -globulin (7.75), BSA (4.27) and myoglobin (2.04); their positions in the gradient were determined by Bio-Rad protein assay at 595 nm or at 410 nm to measure specifically the position of myoglobin. To estimate the molecular mass of TK from the $s_{20,w}$ value the following formula was applied: $s_{20,w} = 0.00242M^{0.67}$, where *M* is the molecular mass.

Incorporation of [3 H]dT *in vivo* into DNA of *T. vaginalis*

T. vaginalis cells, grown as described above, were centrifuged and resuspended at a density of 2×10^6 cells/ml in CPLM medium, modified as follows to obtain a thymidine-free medium: 0.6 % NaCl/0.01 % sodium bicarbonate/0.01 % KCl/0.01 % CaCl_2 (Ringer's solution) and 1 % Bacto-casamino acid (Difco)/0.24 % cysteine hydrochloride/0.2 % maltose. Cells were then incubated for 15 min at 37 °C. Then [3 H]dT (25 Ci/mmol) was added to a concentration of 10 μ Ci/ml. Incubation was then continued in the presence of different concentrations of MTdU and ITdU, and, at 15, 30 and 45 min, 0.08 ml samples of culture were spotted on 25 mm GF/C (Whatman) filters. Trichloroacetic acid-insoluble material was determined as previously described [12].

Antiprotozoan activity of MTdU and ITdU *in vivo*

Serial dilutions of the compounds under test were added to 2 ml *T. vaginalis* cultures (4×10^3 cells/ml) in CPLM medium. Tubes were incubated at 37 °C in the presence of MTdU and ITdU; after 48 h the cell cultures were observed under the microscope and cells were counted on a haemocytometer.

To determine the viability of *T. vaginalis* and the minimal lethal dose of MTdU and ITdU against *T. vaginalis*, 0.4 ml of the previous cultures was removed from the tubes and inoculated in 1.6 ml of fresh CPLM medium. After 48 h subcultures were observed and counted; the presence of viable (motile) parasites was determined.

Measurement of human cell viability: dye-exclusion assay

HeLa cells S-3, grown in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal calf serum, were incubated at 37 °C in air/CO₂ (19:1) at concentrations of 8000 or 80000 cells/ml for 48 h in screw-capped Eppendorf tubes in the presence of different concentrations of the compounds under test. Cells were then centrifuged at 50 *g* and resuspended in 100 μ l of PBS. Trypan Blue solution (100 μ l; 0.4 % in PBS) was added and viable cells (unstained) were counted. Each point was done in duplicate and the number of cells counted in each tube is the average of five determinations. To determine the extent of cell growth after exposure to the compounds for 48 h, a parallel experiment was performed in which treated cells were centrifuged, washed with PBS and incubated for further 24 h in fresh medium and counted as described above.

RESULTS

Identification and purification of TK activity in *T. vaginalis*

We first tried to determine the presence of a thymidine-phosphorylating activity in *T. vaginalis* by assaying the crude extract of the parasite, prepared as described in the Experimental section, for both TK and NP activities. The NP activity was described and purified by Wang and Cheng [13] as a non-specific NP activity, which phosphorylates the four deoxyribonucleosides to their respective monophosphates. The presence of TK activity in a crude extract was reported by Miller [3] but its purification was never attempted. We found both TK (30 units) and NP (625 units) activities in the crude extract and we purified *T. vaginalis* TK as summarized in Table 1. During the purification, NP activity was also assayed. TK activity, which increased after the DEAE-Sepharose column, was purified 1000-fold on a thymidine 3'-phosphate-CH-Sepharose affinity column. The enzyme preparation very rapidly lost its activity: BSA was then added to the elution buffer of the affinity column to prevent the inactivation of the enzyme. Half of the NP activity was lost in the S-100 pellet. The remaining activity was separated from TK on DEAE-Sepharose and recovered in the non-adsorbed fraction (results not shown).

T. vaginalis TK: catalytic properties

Purified *T. vaginalis* TK requires Mg²⁺ ions for its catalytic activity with an optimal range between 4 and 6 mM (results not shown) and is capable of transferring phosphate from ATP but not from other phosphate donors such as acetylphosphate, phosphoribosylpyrophosphate or dUMP. The Lineweaver–Burk plot shown in Figure 1(A) demonstrates that *T. vaginalis* TK phosphorylates [3 H]dT with a K_m of 0.18 μ M (Table 2). To evaluate whether *T. vaginalis* TK is also able to recognize dC, the enzyme was incubated in the presence of various concentrations of substrate [3 H]dT and increasing concentrations of dC. Our results, shown in Figure 1(B), demonstrate that the protozoan TK activity is competitively inhibited by dC with a K_i of 4.4 μ M, indicating that dC enters the active site of the enzyme. To determine whether dC could also be a substrate for the protozoan enzyme we incubated the enzyme with increasing concentrations of [3 H]dC and obtained the plot shown in Figure 1(C), which indicates that the enzyme efficiently phosphorylates dC with a K_m of 0.88 μ M (Table 2). Moreover, the enzyme also phosphorylates dU with an efficiency comparable to that for dT (Table 2) but does not phosphorylate uridine, showing a pyrimidine deoxyribonucleoside kinase activity.

T. vaginalis TK is enantiospecific and lacks an associated thymidylate kinase activity

We have demonstrated that herpes virus TKs [14,15], human deoxycytidine kinase [16] and mitochondrial TK [17] are not enantiospecific and are able to phosphorylate, besides their natural substrates, the respective L-enantiomers. We therefore studied the enantiospecificity of the protozoan TK. Our results demonstrate that *T. vaginalis* TK does not recognize L-dT (results not shown), indicating that the protozoan enzyme is strictly enantiospecific.

To verify whether *T. vaginalis* TK possesses an associated thymidylate kinase activity, as observed for herpes simplex type 1 TK, we incubated *T. vaginalis* TK with [14 C]TMP and analysed the reaction products as described in the Experimental section. No detectable amounts of [14 C]dTDP were revealed even after 1 h of incubation of TK in the presence of [14 C]dTMP, whereas herpes simplex type 1 TK phosphorylated 25 % of the substrate

Table 1 Purification of *T. vaginalis* TK

Purification step	Protein (mg)	Units (nmol/h)	Specific activity (units/mg)	Yield (%)	Purification (fold)
1. Crude extract	450.00	30.0	0.06	100	1
2. S-100	305.00	30.5	0.10	101	1.6
3. DEAE-Sepharose	75.00	52.0	0.69	173	11.5
4. Thymidine affinity chromatography	< 0.05	33.6	672.00	112	11200.0

Figure 1 Determination of kinetic parameters of *T. vaginalis* for dThd and dCyd

(A) Lineweaver-Burk plot for the determination of the K_m of *T. vaginalis* TK for dT. (B) Lineweaver-Burk plot showing the effect of different concentrations of dC on the activity of *T. vaginalis* TK in the presence of increasing concentrations of [3H]dT. dC concentrations were: ●, 0 μM ; ○, 1 μM ; ■, 2 μM ; ▲, 4 μM . Each point is the average of three determinations. (C) Lineweaver-Burk plot for the determination of the K_m of *T. vaginalis* TK for dC.

Table 2 Kinetic parameters of *T. vaginalis* TK

Data are presented as means $\pm$ S.D.

Substrate	K_m (μM)	V_{max} (pmol/h)	V_{max}/K_m (pmol $\cdot$ h $^{-1}$ $\cdot$ μM^{-1})
dT	0.18 ± 0.02	9.2 ± 0.2	51
dC	0.88 ± 0.1	6.2 ± 0.17	7
dU	0.14 ± 0.02	12.9 ± 0.11	92

Figure 2 SDS/PAGE [10% (w/v) gel] of *T. vaginalis* TK stained with silver stain (Merck)

Lane M, molecular mass markers (indicated at the left); lanes 1–4, 0.3, 0.6, 1.5 and 3 μg of BSA respectively; lanes 5–8, 1, 2, 5 and 10 μl of *T. vaginalis* TK preparation containing 0.3, 0.6, 1.5 and 3 μg of BSA respectively.

under the same assay conditions (results not shown). Therefore we conclude that, under our assay conditions, *T. vaginalis* TK does not possess an associated thymidylate kinase activity.

Subunit structure of *T. vaginalis* TK

The molecular mass of the *T. vaginalis* TK was determined on the final enzyme preparation by SDS/PAGE [10% (w/v) gel] stained with silver. As shown in Figure 2, the purified TK preparation, stabilized by the addition of 0.3 mg/ml BSA (band at 66 000 Da), presents a band visible at a position corresponding to a molecular mass of 31 500 Da. However, when analysed by glycerol density ultracentrifugation a single peak of TK activity with an $s_{20,w}$ of 5.9 was observed (results not shown), corresponding to a molecular mass of 113 000. This suggests an association of the 31 500 Da monomer to form multimers. Unfortunately, attempts to dissociate the multimer at high ionic strength (0.5 M potassium phosphate buffer, pH 7, or 1 M KCl), followed either by gel filtration on a Superdex 75 column (Pharmacia) or glycerol density gradient centrifugation, led to inactivation of the enzyme.

Table 3 Effect of nucleoside analogues on *T. vaginalis* TK

Abbreviation: n.i., no inhibition up to 200 μ M, the highest concentration tested. Data are presented as means $\pm$ S.D.

Compound	IC ₅₀ (μ M)	
	<i>T. vaginalis</i> TK	Human TK
MTdU	0.6 $\pm$ 0.05	6.2 $\pm$ 0.7
5-Ethyl-2'-deoxyuridine	21.0 $\pm$ 2.5	n.i.
5-Ethyl-4'-thio-2'-deoxyuridine	13.8 $\pm$ 1.8	n.i.
5-(Cyclopropyl)-2'-deoxyuridine	2.9 $\pm$ 0.35	40.0 $\pm$ 5.1
5-Cyclopropyl-4'-thio-2'-deoxyuridine	6.6 $\pm$ 0.9	60.0 $\pm$ 5.7
5-(Isopropyl)-2'-deoxyuridine	n.i.	n.i.
5-Isopropyl-4'-thio-2'-deoxyuridine	n.i.	n.i.
IdU	0.5 $\pm$ 0.05	6.0 $\pm$ 0.4
ITdU	0.7 $\pm$ 0.04	21.0 $\pm$ 2.2

Effect *in vitro* of some nucleoside analogues on *T. vaginalis* TK activity

Several non-substrate inhibitors of HSV-1 TK [18–23] (such as N²-substituted phenyldeoxyguanosines, N²-substituted phenylguanines and N²-substituted anilino-uracil and related compounds) and a potent substrate inhibitor (5-bromovinyl-2'-deoxyuridine) were assayed as inhibitors for *T. vaginalis* TK activity *in vitro*. None of them showed any activity against the protozoan enzyme (results not shown). However, when a novel

Table 4 Effect of nucleoside analogues on [³H]dT incorporation into DNA of *T. vaginalis* and HeLa cells

Data are presented as means $\pm$ S.D.

Compound	IC ₅₀ (μ M)	
	<i>T. vaginalis</i>	HeLa
MTdU	9.4 $\pm$ 1.2	4.3 $\pm$ 0.5
ITdU	38.0 $\pm$ 4.5	33.6 $\pm$ 4.2
IdU	25.0 $\pm$ 3.2	4.4 $\pm$ 0.6

series of 4'-thio-2'-deoxyuridine analogues was tested against protozoan and human TKs we found some compounds that preferentially inhibited the protozoan TK (Table 3). Nevertheless it is interesting to observe that, as far as the substituent at C-5 is concerned, the specificity of *T. vaginalis* TK is very similar to that of human TK, i.e. CH₃ > I > cyclopropyl > ethyl.

Those inhibitors with the best selectivity index were two 4'-thio-derivatives (MTdU and ITdU), which were required at approx. 10–30-fold higher concentration to inhibit the human TK by 50 %. MTdU and ITdU inhibit [³H]dT phosphorylation with IC₅₀ values of 0.6 and 0.7 μ M respectively; obviously they also inhibit the phosphorylation of [³H]dU and [³H]dC.

Insight into the mechanism by which MTdU and ITdU inhibit *T. vaginalis* TK was sought by studying the dependence of their inhibition on the concentration of the substrate in the reaction.

Figure 3 Lineweaver–Burk plots (A, C) and Cornish–Bowden plots (B, D) showing the effect of different concentrations of MTdU and ITdU on the activity of *T. vaginalis* TK in the presence of increasing concentrations of [³H]dT (dThd)

(A) MTdU concentrations were: ●, 0 μ M; ○, 0.02 μ M; ■, 0.04 μ M; ▲, 0.06 μ M. (C) ITdU concentrations were: ●, 0 μ M; ○, 0.03 μ M; ■, 0.06 μ M; ▲, 0.09 μ M. (B, D) [³H]dT concentrations in Cornish–Bowden plots were: ●, 0.1 μ M; ○, 0.15 μ M; ■, 0.3 μ M; ▲, 0.6 μ M. Each point is the average of three determinations.

Figure 4 Effect of MTdU (●) and ITdU (□) on proliferation of *T. vaginalis*

T. vaginalis was inoculated at 4000 cells/ml under the conditions described in the Experimental section; the cell numbers shown on the ordinate were determined after 48 h of exposure to the compounds. Points represent the average of three experiments; the S.D. for each point was less than 15%.

The effect of these compounds at different concentrations of dT is displayed in the double-reciprocal plot, shown in Figures 3(A) and 3(C), allowing the determination of K_i values of 20 and 24 nM for MTdU and ITdU respectively. The kinetic analysis of

these results demonstrate that the inhibition is competitive with the substrate [see the Cornish-Bowden plots of $[S]/v$ against $[I]$ shown in Figures 3(B) and 3(D), where the essentially parallel lines indicate a competitive inhibition]. This suggests a direct binding of the compounds to the catalytic site of the enzyme.

Finally, these compounds (tested up to 250 μ M) did not inhibit an ATP-dependent uridine-phosphorylating activity present in *T. vaginalis* (S-100 fraction; results not shown), confirming that uridine kinase and TK are different enzymes [3].

MTdU and ITdU are phosphorylated by *T. vaginalis* TK

To understand whether MTdU and ITdU are substrate inhibitors of the protozoan enzyme or are pure non-substrate inhibitors, we incubated *T. vaginalis* TK with either MTdU or ITdU in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$; the resolution of reaction products was performed by reverse-phase chromatography as described in the Experimental section. Under the above assay conditions, dT, used as control, MTdU and ITdU were phosphorylated with similar efficiencies (results not shown).

Effect of 4'-thio-2'-deoxyuridine analogues on thymidine incorporation into DNA of *T. vaginalis*

To verify the ability of MTdU, ITdU and 5-iodo-2'-deoxyuridine (IdU) (used as control) to interfere with $[\text{H}^3]\text{dT}$ incorporation

Figure 5 Effect of MTdU (A, B) and ITdU (C, D) on growth and viability of HeLa S3 cells

Cell viability was measured by a dye exclusion assay as described in the Experimental section. HeLa S3 cells (1 ml) were incubated at 8000 cells/ml (A, C) or at 80 000 cells/ml (B, D) in the presence of different concentrations of the compounds (zero time; open bars). After 48 h viable cells were counted (48 h; stippled bars). In parallel, cells exposed for 48 h to the compounds were washed with PBS and fresh medium was added. After 24 hr the cells were counted (48 + 24 h; tinted bars).

into protozoal DNA, *T. vaginalis* cells were incubated in the presence of [³H]dT and increasing concentrations of the compounds as described in the Experimental section. MTdU, ITdU and IdU strongly inhibited [³H]dT incorporation into *T. vaginalis* DNA with IC₅₀ values of 9.4, 38 and 25 μ M respectively. The possible inhibitory effect of MTdU, ITdU and IdU on [³H]dT incorporation was also tested in HeLa cells. In this system MTdU, ITdU and IdU decreased [³H]dT incorporation with IC₅₀ values of 4.3, 33.6 and 4.4 μ M respectively (Table 4). Comparison of the results in Table 4 with those in Table 3 shows that the concentrations of thionucleosides requested to inhibit [³H]dT incorporation in cultured *T. vaginalis* cells were higher than those expected from their IC₅₀ *in vitro* on purified *T. vaginalis* TK. In contrast, the concentrations required to inhibit [³H]dT incorporation in HeLa cells were similar to those required to inhibit purified TK *in vitro* by 50%. Because human cells contain only a thymidine-phosphorylating activity (TK) and *T. vaginalis* contains two (TK and NP, the latter being the predominant activity), this suggests that NP is resistant to the thionucleosides. Indeed, we found that this latter enzyme was fully resistant to MTdU and ITdU (tested up to 250 μ M; results not shown). However, we cannot completely exclude the possibility of a lower uptake of inhibitors by *T. vaginalis* cells.

Effect of MTdU and ITdU on proliferation of *T. vaginalis*

The inhibition of protozoan DNA synthesis by MTdU and ITdU suggested that it would be worth evaluating their effect on *T. vaginalis* cultures. Serial dilutions of the compounds were added to 2 ml of *T. vaginalis* culture. Figure 4 shows that MTdU and ITdU are cytotoxic for *T. vaginalis* with IC₅₀ values of approx. 7.5 and 24 μ M respectively. Furthermore we have observed that at inhibitor concentrations that inhibited the cell proliferation by less than 50%, *T. vaginalis* were clearly suffering, as demonstrated by their decreased motility. To determine whether the parasites were able to recover after 48 h of exposure to high doses of MTdU and ITdU, a 0.4 ml sample was removed from each tube and inoculated in 1.6 ml of fresh CPLM medium. After 48 h, *T. vaginalis* cells present in the subcultures were counted and no viable (motile) parasite was observed in the subcultures derived from the cultures preincubated in the presence of MTdU and ITdU at 100 μ M (minimal lethal dose; results not shown) or higher concentrations, as determined by the procedure described in the Experimental section.

Effect of MTdU and ITdU on human cell growth and viability

To evaluate the effects of MTdU and ITdU on human cells growth and viability we exposed HeLa cells (8000 and 80000 cells/ml) to various concentrations of the compounds. Cell viability was measured by the dye exclusion assay described in the Experimental section.

Figure 5 shows that MTdU and ITdU up to 50 μ M affect the growth of HeLa cells, exerting only a cytostatic effect. In fact cells overcame the cytostatic effect when the medium containing the inhibitor was removed and replaced with fresh medium. At higher concentration (100 μ M) only MTdU was cytotoxic both at low cell concentrations (Figure 5A, 48 h bar) and at high cell concentrations (Figure 5B, 48 h bar), whereas ITdU was cytotoxic only at low cell concentrations (Figure 5C, 48 h bar).

DISCUSSION

This work reports the first purification of a TK activity from *T. vaginalis*. The enzyme phosphorylates not only dT but also dC and dU, but not U, to the respective monophosphates, showing

a typical pyrimidine deoxyribonucleoside activity. Because of the lack of the synthesis of pyrimidine and purine nucleotides *de novo* [1,2] and the lack of ribonucleotide reductase, which converts ribonucleotides to deoxyribonucleotides [13], the parasite bases its requirement for dTTP and dCTP for DNA synthesis on salvage pathways, but it was previously still unclear whether NP [13] or deoxyribonucleoside kinases [3], or both, are involved. This work demonstrates that both TK and NP are present in *T. vaginalis* extracts and reports the first purification of TK. TK was separated from NP by ultracentrifugation and ion-exchange chromatography and purified to apparent homogeneity by affinity chromatography. The enzyme, probably a tetramer of 113000 Da, catalytically resembles cytoplasmic human TK because of (1) its resistance to several nucleoside and non-nucleoside inhibitors of herpes virus TK [18–23]; (2) its enantiospecificity; (3) the absence of an associated thymidylate kinase activity; and (4) the parallel decreasing inhibitory effect caused by the substituent at C-5 (CH₃ > I > cyclopropyl > ethyl).

However, it is 10–30-fold more sensitive to the novel deoxyuridine analogues MTdU and ITdU than is the human enzyme. MTdU and ITdU are efficiently phosphorylated by *T. vaginalis* TK and inhibit [³H]dT incorporation in cultured *T. vaginalis* cells with IC₅₀ values (7.5 and 24 μ M respectively) higher than those required to inhibit the purified *T. vaginalis* TK by 50%. Also when tested on *T. vaginalis* growth, the thionucleosides present IC₅₀ values 10–30-fold the concentrations inhibiting purified *T. vaginalis* TK. This is probably because an NP activity, totally resistant to MTdU and ITdU, is also present in the protozoan cell and contributes 20-fold more than TK to the formation of dTMP. Therefore these compounds can interfere with *T. vaginalis* DNA replication by the depletion of dTMP or dCMP; alternatively and more probably, once in the triphosphate form they interfere directly with DNA replication by inhibiting protozoan DNA polymerase. Although the inhibitors described here are also cytotoxic at high concentrations for human cells, they demonstrate that protozoan TK has a key role in the growth and viability of *T. vaginalis* and therefore that this enzyme can be considered a new target for the design of antitrichomonal drugs.

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