Expression, Purification, Characterization, and in Vivo Targeting of Trypanosome CTP Synthetase for Treatment of African Sleeping Sickness*

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African sleeping sickness is a fatal disease caused by two parasite subspecies: Trypanosoma brucei gambiense and T. b. rhodesiense. We previously reported that trypanosomes have extraordinary low CTP pools compared with mammalian cells. Trypanosomes also lack salvage of cytidine/cytosine making the parasite CTP synthetase a potential target for treatment of the disease. In this study, we have expressed and purified recombinant T. brucei CTP synthetase. The enzyme has a higher $K_m$ value for UTP than the mammalian CTP synthetase, which in combination with a lower UTP pool may account for the low CTP pool in trypanosomes. The activity of the trypanosome synthetase is irreversibly inhibited by the glutamine analogue acivicin, a drug extensively tested as an antitumor agent. There is a rapid uptake of acivicin in mice both given intraperitoneally and orally by gavage. Daily injection of acivicin in trypanosome-infected mice suppressed the infection up to one month without any significant loss of weight. Experiments with cultured bloodstream T. brucei showed that acivicin is trypanocidal if present at $1 \mu M$ concentration for at least 4 days. Therefore, acivicin may qualify as a drug with “desirable” properties, i.e. cure within 7 days, according to the current Target Product Profiles of WHO and DNDi.

There are only two drugs known to be effective against the late stage of the disease, DL-$\alpha$-difluoromethylornithine (DFMO, 3 efornithine) and Melarsoprol. DFMO can only cure T. b. gambiense infections. Furthermore, because of the lengthy infusion schedules, it can only be administered in a hospital setting. Melarsoprol, an old arsenical derivative, also has to be given by infusion but in addition is causing serious side effects such as fatal encephalopathy in as high as 10% of the cases. Furthermore, there is an increasing resistance to Melarsoprol reaching almost 30% in central Africa (2). Because of the highly variable nature of the glycoprotein coat, all attempts to develop an efficient vaccine have met with little success.

CTP pools in trypanosomes were previously demonstrated to be very low compared with other eukaryotic cells and trypanosomes totally lack the ability to salvage cytosine or cytidine (5, 6). CTP is essential for the biosynthesis of nucleic acids and phospholipids (7–9), both necessary for cell survival. For these reasons, T. brucei CTP synthetase, a glutamine amidotransferase responsible for de novo synthesis of CTP, was suggested to be a potential drug target for treatment of African sleeping sickness (6).

CTP synthetase (CTPS) was originally identified 1955 in Escherichia coli by Lieberman, who showed that the enzyme catalyzes the conversion of UTP to CTP in the presence of ATP, ammonia, and magnesium (10). The bacterial enzyme can use glutamine as an alternate nitrogen donor when GTP is present as an allosteric effector (11). ATP and UTP were shown to induce a tetrameric form of the enzyme (12). The partially purified CTPS from calf or rat liver shows strong similarity to the bacterial enzyme but the mammalian enzyme has no absolute requirement for GTP in the presence of glutamine as a substrate (13, 14). The crystal structures of CTPS from E. coli and Thermus thermophilus were recently reported (15, 16) as well as the structure of the synthetase domain of human CTPS (17).

Like other glutamine amidotransferases, CTPS is inhibited by glutamine analogues (18, 19). We showed earlier that DON (6-diazo-5-oxo-L-norleucine) and acivicin ($\alpha$-amino-3-chloro-4,5-dihydro-5-isoxazolacetic acid) reduced the already low parasitic CTP levels even further and inhibited T. brucei growth in vitro (6). Addition of acivicin to cultured bloodstream T. brucei specifically lowered the parasitic CTP and GTP pools without affecting the ATP and UTP pools indicating that the main

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2 The abbreviations used are: DFMO, DL-$\alpha$-difluoromethylornithine; CTP, CTP synthetase; DTT, dithiothreitol; GEMMA, gas-phase electrophoreticmobility macromolecule/nanoparticle analysis; TEV, tobacco etch virus.
targets in the parasite are CTPS and GMP synthetase like in mammalian cells. Addition of guanine, cytosine and cytidine to the growth medium restored the GTP pool but neither restored the CTP pool nor growth. Acivicin is potentially a better drug candidate than DON since it penetrates the blood-brain barrier and has a longer in vivo half-life (20–22). Acivicin was isolated from cultures of Streptomyces sviceus 1973 and shown to irreversibly inhibit glutamine amidotransferase by alkylating a nucleophilic active site cysteine (23, 24). Pharmacokinetic and toxicity studies in several animal models and in human cancer patients extensively characterized the efficacy and the dose-limiting CNS toxicity of acivicin as an anti-tumor agent (20, 21, 25–28). However, the ability of tumor cells to salvage purines and pyrimidines provided resistance against acivicin treatment and limited its value as an anti-tumor drug (25, 29).

In this study, we have expressed and purified recombinant T. brucei CTPS to homogeneity. Kinetic studies of the pure enzyme indicate that the low CTP pools in trypanosomes are a consequence of the high $K_m$ for UTP compared with the mammalian enzyme. The trypanosome CTPS showed the same strong affinity for acivicin as the rat liver enzyme but preincubation of the trypanosome enzyme in the presence of the nucleotide substrates together with acivicin strengthened the inhibition. Our in vivo experiments demonstrate that it is possible to suppress trypanosome infections in mice for at least one month periods using acivicin without serious side effects. Experiments with cultured bloodstream T. brucei in vitro showed that acivicin has a trypanocidal effect if present in 1 $\mu$M concentration for at least 4 days. This concentration is well below the concentration of 5 $\mu$M in human plasma where CNS toxicity starts to appear if the drug is present more than 16 h (27). Analyses of acivicin levels in mouse blood showed that oral administration of drug results in almost the same rapid uptake as intraperitoneal administration with a half-life of about 72 min increasing its potential value for treatment of African sleeping sickness.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The T. brucei CTPS gene was amplified from genomic DNA of the TC221 strain using primers with the following sequence: 5’-AGTTAAGCGGATAGCAGGAGGAG-3’ and 5’-ACACATATATACACTAGACTCGTT-3’. Using the diluted (100×) PCR product as a template, a second round of PCR was performed. This time one of the primers contained an NdeI recognition sequence followed by a His$_6$ coding region and a tobacco etch virus protease recognition sequence preceding the start codon: 5’-CATATGGCATCCACCTACCACATCGATTACGATATCCCAACGAGCAGAACCATGCGTAGGCATCCGATGCCACGTCGCACTCCAGACTCCAG-3’. The reverse primer contained an EcoRI recognition sequence: 5’-GAAATTCGATATCCGATGCCACGTCGCACTCCAGACTCCAG-3’. Upon digestion of the PCR product with EcoRI and NdeI, the DNA was cloned into a pETM2 vector replacing the M2cDNA (30).

**Expression and Purification of Recombinant T. brucei CTP Synthetase**—His$_6$-TEV protease site-tagged T. brucei CTP synthetase was expressed in BL21(DE3) pLysS bacteria. The cells were grown at 15°C in Terrific Broth medium supplemented with 84 $\mu$M chloramphenicol (USB) and 118 $\mu$M carbenicillin (Duchefa Biochemie) until A$_{600}$ ≈ 12. The bacteria were harvested by centrifugation for 20 min at 4000 $\times$ g at 4°C. The cell pellet was washed with ten volumes of 50 mM Tris-Cl, pH 7.6, 100 mM NaCl, 1 mM EDTA and after centrifugation (as above) resuspended in two volumes of 50 mM Hepes buffer pH 7.3, 0.2 M NaAc. The cells were disrupted by three cycles of freezing and thawing. The crude extract was centrifuged in an ultracentrifuge for 1 h at 150,000 $\times$ g at 4°C.

The supernatant was added to a Talon Metal Affinity resin (Clontech) equilibrated with 50 mM Hepes buffer pH 7.3, 0.2 M NaAc and incubated for 1 h at 4°C on a rotary platform. The resin was pelleted by centrifugation at 2000 $\times$ g for 4 min at 4°C and washed once with 10 resin volumes of the same buffer. After repelleting, the Talon resin was washed twice with 50 mM Hepes buffer, pH 7.3, 0.2 M NaAc supplemented with 5 mM imidazole. Following the second imidazole wash, the resin was packed into a glass Econo-Column (Bio-Rad), and the CTPS was eluted from the column with 50 mM Hepes buffer pH 7.3, 0.2 M NaAc to remove the imidazole, and the protein-containing fractions were concentrated in a 10-kDa Vivaspin 6 concentrator (Viva Science) at 2000 $\times$ g. A Ni-NTA purified His$_6$-TEV protease (the pET9d construct was kindly provided by Günther Stier, EMBL, Heidelberg) was added to the concentrated solution, and proteolysis was performed at 23°C for 40 min. The protein solution was then loaded again on a Talon resin column but this time the flow-through, containing CTPS without His tag, was collected. Finally a 0.2-nl Source Q 15 medium column (Amersham Biosciences) was packed and equilibrated with 50 mM Hepes buffer pH 7.3, 0.2 M NaAc. The protein solution was loaded onto the column which was eluted with 5 column volumes of the same buffer. The flow through fractions containing T. brucei CTPS were collected and pooled. After the addition of
EDTA to a final concentration of 1 mM, the protein solution was frozen and stored at −70 °C.

Standard CTPS Activity Assays and Protein Determination—Protein concentrations were determined by the Bio-Rad protein assay. The standard activity assay mixture consisted of 0.1–1 μg of CTPS in 50 μl of reaction buffer (0.2 mM KCl, 20 mM dithiothreitol (DTT), 30 mM MgCl₂, 0.4 mM GTP, 4 mM [³H]UTP (specific activity 18,000 cpnm/mmol), 8 mM ATP, 5 mM L-glutamine, and 50 mM Hepes, pH 7.3). Before the start of the reaction by the addition of L-glutamine, the reaction mixtures were preincubated at 37 °C for 1 min. The reactions were run for 5 min at the same temperature, stopped by the addition of 0.5 ml of 1 M perchloric acid and incubated on ice for 10 min. Before centrifugation at 21,000 x g for 10 min, to remove the precipitated proteins, 250 nmol of carrier CTP were added. The supernatants were boiled in a water bath for 10 min to hydrolyze the nucleotides to monophosphates and then cooled on ice. The samples were neutralized by KOH and centrifuged for 2 min at 21,000 x g at room temperature. Finally, the supernatants were loaded onto glass Econo-columns packed with 4 ml of Dowex 50 W×8, 400 mesh cation exchange medium (Sigma Aldrich). The columns were first washed with 20 ml of 1 M HCl followed by a 12 ml wash with 0.2 M HCl. The CMP was eluted in an additional 5 ml of 0.2 M HCl and the concentration determined from the absorbance at 280 nm. The amount of nucleotides was determined by a standard enzyme reaction as a function of glutamine concentration at 30 °C. The concentration of the drug was estimated from standard curves by comparing the inhibition zones to those obtained by samples with known concentration.

Inhibition of T. brucei CTPS Activity by Acivicin—Inhibition by acivicin was determined by studying the initial velocity of the enzyme reaction as a function of glutamine concentration at various acivicin concentrations. Using the standard reaction mixture, acivicin was added at the same time as L-glutamine. To be able to compare the inhibition by acivicin of T. brucei CTPS with the published value of the mammalian CTPS, the Kᵦ value for acivicin was obtained from a Dixon plot (31). The preincubation experiments were performed as follows: Recombinant T. brucei CTPS (0.25 μg/μl) in 50 mM Hepes buffer pH 7.3, 0.2 mM KCl was incubated in the presence or absence of 12 μM acivicin, 8 mM ATP, 30 mM MgCl₂, 0.4 mM GTP, and 4 mM [³H]UTP. The mixtures were preincubated from 1 to 5 min at 37 °C, and then 2 μl transferred to 48 μl of preincubated standard reaction mixture containing 5 mM L-glutamine. The reactions were stopped after 5 min and processed as above. The same results were obtained with and without DTT in the preincubation mixture.

**Table 1** Purification of T. brucei CTP synthetase

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Crude extract</th>
<th>1st Talon eluate</th>
<th>TEV-cleaved 2nd Talon flow-through</th>
<th>Concentrated SourceQ flow-through</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>792</td>
<td>429</td>
<td>287</td>
<td>221</td>
</tr>
<tr>
<td>Protein amount (mg)</td>
<td>1200</td>
<td>3.6</td>
<td>0.72</td>
<td>0.4</td>
</tr>
<tr>
<td>Specific activity (units/mg)</td>
<td>0.66</td>
<td>119.2</td>
<td>398.6</td>
<td>551.5</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>100</td>
<td>54.2</td>
<td>36.2</td>
<td>27.9</td>
</tr>
<tr>
<td>Purification (fold)</td>
<td>1</td>
<td>181</td>
<td>604</td>
<td>836</td>
</tr>
</tbody>
</table>

**Table 2** Kₘ values of T. brucei and calf liver CTPS

<table>
<thead>
<tr>
<th>Ligand</th>
<th>T. brucei Kₘ</th>
<th>Calf liver Kₘ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>0.07</td>
<td>0.91</td>
</tr>
<tr>
<td>UTP</td>
<td>0.16</td>
<td>0.07</td>
</tr>
<tr>
<td>GTP</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>0.26</td>
<td>0.21</td>
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</tbody>
</table>

*See Ref. 14.*

**Acivicin Treatment of T. brucei-infected Mice and Acivicin Plasma Measurements—**T. brucei (TC221) infected C57Bl/6 male mice weighing 30 g were treated with daily intraperitoneal (intraperitoneal) injections of acivicin. Alternatively, the mice were given a daily 10 mg/kg acivicin dose per os in the drinking water assuming a daily intake of ~5 ml of water. The levels of trypanosomes in the blood were followed by inspection of blood samples with a microscope with a detection limit of 0.1 million trypanosomes/ml. Acivicin plasma concentrations in mice were measured by adding 15 μl of plasma to paper filters (Oxoid) and placing them on Bacillus subtilis plated on complete synthetic medium plates (23). Zones of inhibition were measured after 15–18 h incubation at 37 °C. The concentration of the drug was estimated from standard curves by comparing the inhibition zones to those obtained by samples with known acivicin concentrations (23, 35).

**Effects of Acivicin on Cultured Bloodstream T. brucei—**T. brucei (TC221) parasites were propagated at 37 °C in 7% CO₂ in a humified atmosphere in Hirumi’s modified Iscove’s medium-9 (36) containing 0.4 mM L-glutamine and 10% fetal calf serum. In a series of tubes, 2 ml of culture containing around 200,000 trypanosomes/ml were incubated with 1–5 μM acivicin for 72 or 96 h. Then the cells were collected by centrifugation at 1500 × g for 3 min, washed once with phosphate-buffered saline and finally resuspended in fresh HMI-9 medium without drug. During the experiment, trypanosome levels were monitored by counting the cells in a microscope with a detection limit of 1000 cells/ml.
Expression and Purification of Recombinant T. brucei CTPS—
To avoid formation of inclusion bodies, the His-tagged CTPS was expressed without IPTG induction. Furthermore, the bacteria had to be grown at 15 °C to maximize the expression of the soluble form of the recombinant enzyme. The low expression levels of soluble protein required the use of metal affinity chromatography on a Co²⁺-bearing Sepharose medium called Talon resin. The second Talon chromatography step was preceded by the removal of imidazole on a Sephadex G-50 column and cleavage of the histidine tag by tobacco etch virus protease which recognizes a sequence consisting of seven amino acids, EXXYXQ (S/G) (37). A shift in mobility of the T. brucei CTPS seen on the SDS-PAGE confirmed the successful proteolysis (Fig. 1). By loading the protein solution a second time onto the Talon resin, we removed the His-tagged TEV protease together with most of the impurities eluted from the first affinity step while we collected the purified CTPS in the flow-through fraction. As a final purification step, a strong anion exchanger, Source Q 15 Sepharose, was employed. Again, the flow-through fraction contained highly purified, recombinant CTPS leaving most of the impurities bound to the column (Fig. 1). During the purification, the specific activity of the enzyme preparation increased 836-fold and the total yield was 28% (Table 1).

Enzyme Kinetics and Acivicin Inhibition—The specific activity of the pure enzyme was determined to 550 units/mg, a value, which is close to the specific activity of the highly purified rat liver enzyme assayed under similar conditions (38). In an attempt to explain the low CTP pools in trypanosomes, we examined the ligand $K_m$ values of the trypanosome CTPS (Table 2). Two striking differences between the published values of mammalian CTPS and the values of the trypanosome enzyme were observed. (i) The $K_m$ of the trypanosome enzyme for UTP is two times higher compared with the corresponding value for the mammalian CTPS (0.16 mM and 0.07 mM, respectively) (14). (ii) The $K_m$ of T. brucei CTPS for ATP is much lower than the corresponding value for the mammalian CTPS (0.07 mM compared with 0.91 mM). It appears as even though the velocity of the reaction is increased 7-fold when the trypanosome enzyme is saturated with ATP, the enzyme shows no absolute requirement for ATP when all the other four ligands are present at saturating concentrations. Most probably, ATP can be substituted against GTP, without which there is no reaction (Fig. 2).

The conditions and methods for measuring the inhibition constant of acivicin were similar to those of Neil et al. (31) to make the comparison to the mammalian CTPS as valid as possible. From the Dixon plot, where the inverted initial velocities were plotted as a function of acivicin concentration at a series of increasing glutamine concentrations, we could determine the $K_i$ of the trypanosome CTPS for acivicin to 2.3 μM (Fig. 3). As observed for other glutamine amidotransferases (39), the acivicin inhibition of T. brucei CTPS was more pronounced when the enzyme was preincubated with the drug in the presence of nucleotide substrates than in the absence of substrates (Fig. 4).
Oligomeric Structure Studies of T. brucei CTPS—There has been lots of discussion in the literature about the oligomeric structure of enzymatically active CTPS from different species (12, 13, 40, 41). In the case of trypanosome CTPS, we took advantage of the new GEMMA technique, which allows direct determination of the oligomeric forms of non-covalent protein complexes at the low concentration of protein used in the assay.

From the GEMMA experiments, we could observe that at an enzyme concentration of 20 μg/ml, which is similar to the concentration in the activity assay, the dominating forms of the trypanosome CTPS in the absence of nucleotides were monomers and tetramers. The concentration of dimers was about half of the monomer or tetramer concentration (Fig. 5A). After the addition of 20 μM ATP or UTP alone or in combination, the balance strongly shifted toward the tetrameric form (Fig. 5, B–D) indicating that the active form of the trypanosome CTPS is a tetramer.

Acivicin Treatment of Non-infected and T. brucei-infected Mice—From previously published data (31, 35) and our own toxicity studies (Fig. 6) we could observe that at a daily intraperitoneal dose of 10 mg/kg acivicin in male mice, the animals gradually lost their weight suggesting severe side effects. On the other hand, a daily dose of 5 mg/kg (Fig. 6) or less (data not shown) did not cause any observable weight loss during the same period. In non-infected mice, the leukocyte count did not show any significant change after injection of 1 mg/kg for 12 days. Infection rapidly decreased the leukocyte count from around 12 × 10⁶ cells/ml to 5 × 10⁶ cells/ml but after the daily injections of 5 mg acivicin/kg for 20 days the leukocyte count was still around 2.5 × 10⁶ cells/ml (data not shown).

Typically, intraperitoneal injection of 2 × 10⁵ trypanosomes of the strain TC221 in a mouse resulted in a rapid increase in trypanosomes in the blood, and the mouse died after 4 days with around 200 × 10⁶ trypanosomes/ml (Fig. 7A). When treated with daily intraperitoneal injections of 5 mg/kg acivicin starting at day 5 postinfection and lasting for 30 days, the trypanosomes in the blood rapidly decreased from around 50 × 10⁶/ml to below the level of detection (0.1 × 10⁶ trypanosomes/ml) and no trypanosomes could be detected in the blood during the period of treatment (Fig. 7B). Furthermore, the mouse did not lose weight during the treatment. However, a few days after stopping the acivicin injections, the trypanosomes reappeared in the blood. A shorter treatment period of 17 days with the same dose of acivicin resulted in trypanosomes reoccurring 3 days after the last dose of the drug (data not shown). A similar outcome was obtained when a mouse was infected with the lower dose of 2 × 10⁴ trypanosomes and treated with daily intraperitoneal injections of 5 mg/kg acivicin for 6 days. The trypanosomes in the blood disappeared but 5 days after the last acivicin injection they started to reappear again (Fig. 7C).
A microbiological assay of *B. subtilis* growth inhibition was used to investigate if the concentrations of acivicin in the blood are too low with only one injection per day to prevent the reoccurrence of the parasites when the treatment is stopped (Fig. 7D). There was a rapid increase in acivicin levels in the blood after intraperitoneal injection and the drug disappeared with a half-life of about 72 min. To allow a more frequent administration of drug, we also tried to give acivicin by gavage as an alternative to intraperitoneal injection. Also after administration by gavage, the increase in blood concentration of acivicin was very similar to the increase observed after intraperitoneal injection and we could not see any difference in the half-life values for acivicin plasma concentrations in infected animals when comparing the two administration routes.

**Acivicin Is Trypanocidal When Given to Cultured Bloodstream T. brucei for a Period of 3–4 Days**—The results with trypanosome-infected mice, where trypanosomes reappeared in the blood soon after the administration of acivicin was discontinued, suggested that acivicin is only trypanostatic. To study the effects of acivicin on trypanosomes more in detail, trypanosomes were cultured in *vitro* in a medium with a concentration of glutamine of 0.4 mM corresponding to the concentration in human plasma (42). As seen in Fig. 8, the number of trypanosomes decreased gradually with time in cultures incubated with 1–5 μM acivicin in strong contrast to cultures incubated without drug. After 72 h of incubation in 1 or 2 μM acivicin, the cultures still contained living parasites as seen from the rapid restitution of growth after removal of the drug. However, no growth was observed after 72 h of incubation in 5 μM acivicin or after 96 h of incubation in 1 or 2 μM acivicin. We conclude that acivicin is indeed trypanocidal if continuously present in the medium for prolonged periods.

**DISCUSSION**

We have previously determined that the unusually low CTP pools in *T. brucei* are a direct result of slow CTP synthesis and not a consequence of increased turnover (6). This finding made the trypanosome CTPS a potential target for the treatment of African sleeping sickness. Addition of cytidine or cytosine to the medium had no effect on the lowered trypanosome CTP pools or growth inhibition after acivicin treatment demonstrating the essential role of CTPS in the absence of salvage (6).

Why do trypanosomes have such low CTP pools? To answer this question, we compared the kinetic data for our pure, recombinant trypanosome CTPS with the published data of a partially purified (about 20% pure) calf liver CTPS (14). The *K_m* for UTP appears to be more than twice as high for the trypanosome CTPS than for its counterpart in calf liver cells (0.16 and 0.07 mM, respectively). This difference becomes even more important when the cellular concentrations of UTP in the organisms are considered: 0.28 mM in *T. brucei* and 0.6 mM in a mammalian fibroblast (5, 43). Therefore, the intracellular pool of UTP may be crucial for CTPS activity in *T. brucei* offering one possible clue to why trypanosomes have such a low CTP pool.

The *K_m* for ATP was more than ten times lower for the *T. brucei* CTPS compared with the reported *K_m* of the calf liver enzyme. However, the *K_m* value for ATP of the *E. coli* CTPS is 0.2 mM (44), similar to the value of the trypanosome CTPS, suggesting that the strikingly high *K_m* of the calf liver enzyme may be explained by the presence of other ATP-hydrolyzing enzymes in the partially purified mammalian enzyme preparation.

In a previous investigation, Hofer et al. (6) showed that the trypanosomes are 80% growth-inhibited upon 15 h of incubation in tissue culture with 1 μM acivicin. According to Neil et al. (26) ~14 times higher acivicin concentration is required to achieve the same growth inhibition of mouse L1210 cells in culture. We show that the *K_s* for the initial binding of acivicin to the purified recombinant *T. brucei* CTPS is 2.3 μM, which is approximately the same value as for the mammalian enzyme (31). However, the greater inhibition by acivicin of trypanosome growth than growth of mammalian cells may be explained by the fact that trypanosomes in contrast to mammalian cells...
Although daily acivicin injections so far did not permanently cure mice from infection with the strain TC221 of trypanosomes, we have been able to suppress the infection in a mouse for one month. Intriguingly, when the drug was given ad libitum for 5 days in the drinking water at a nominal dose of 10 mg/kg, we could in one case observe that the reoccurrence of trypanosomes was postponed 8 days (data not shown). This suggests that the way and frequency of the distribution of acivicin could prove to be decisive for permanent cure of the infection. From our measurements of the concentration of acivicin in the blood (Fig. 6) it is obvious that after one single intraperitoneal injection of 10 mg/kg, the plasma concentrations are above 1 \( \mu M \) for only about 3 h. Hopefully, a more evenly distributed intake of the drug over the course of the day instead of a single injection leading to more constant plasma levels may more efficiently affect the growth of trypanosomes.

This hypothesis is strongly supported by our in vitro data clearly demonstrating the trypanocidal effect of acivicin if given continuously in concentrations of 1 \( \mu M \) for at least 96 h. It is evident from these experiments that the duration of drug in the medium is more important than a high concentration. In phase I clinical trials with acivicin to cure human cancer, the dose-limiting factor was CNS toxicity (27, 28). In these trials, plasma levels at the end of 72 h continuous infusions ranged from 0.5 to 6 \( \mu M \) where CNS toxicity correlated to acivicin levels exceeding 5 \( \mu M \) for more than 16 h. To obtain plasma levels of acivicin of 1 \( \mu M \), a dose of 15–30 mg/m\(^2\) should be administered over a 24-h period, and this is well below the starting dose of 60 mg/m\(^2\) recommended for phase II clinical trials in treatment of solid tumors (27).

It was reported earlier that inhibition of L1210 mouse leukemia cells growth in vitro by acivicin is antagonized by combinations of cytidine and guanosine (31). These nucleosides can be salvaged by mammalian cells to supply CMP and GMP for their nucleotide metabolism. However, trypanosomes are unable to salvage cytosine or cytidine and therefore a combination of acivicin with cytidine and guanosine may lower the side effects of the drug in humans.

An advantage of acivicin compared with Melarsoprol or DFMO may be that the drug can be given orally. In our experiments we could show that acivicin is almost as rapidly absorbed through an intragastric administration by gavage as through an intraperitoneal injection.

With emerging reports of spread of the DFMO resistant \( T. b. rhodesiense \) (45, 46) into the endemic areas of \( T. b. gambiense \) there is an increasing risk of an ineffective treatment of the patients. Furthermore, because eflornithine is now used with increased frequency there is a concern that the parasites will inevitably develop resistance against the drug. Several trials involving combination therapy are currently under evaluation (47). Much like DFMO, acivicin has already undergone extensive clinical trials as an anti-cancer drug and the toxicity in human patients and mechanism of action is well characterized. If our in vitro results are valid also in vivo, acivicin would classify as a drug with “desirable” properties, i.e. complete cure within 7 days, according to the current Target Product Profiles of WHO and DNDi.