The Adenosine Analog Tubercidin Inhibits Glycolysis in *Trypanosoma brucei* as Revealed by an RNA Interference Library*

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We used a RNA interference (RNAi) library in a forward genetic selection to study the mechanism of toxicity of tubercidin (7-deazaadenosine) to procyclic *Trypanosoma brucei*. Following transfection of cells with an RNAi-based genomic library, we used 5 μM tubercidin to select a drug-resistant cell line. Surprisingly, we found in these resistant cells that the hexose transporters had been silenced. We subsequently found that silencing of hexokinase, a glycolytic enzyme, also yielded tubercidin-resistant parasites. These observations suggested that glycolysis could be a target of tubercidin action and that RNAi silencing of glycolytic enzymes was gradual enough to allow the parasites to adapt to alternative sources of energy. Indeed, adaptation of procyclic trypanosomes to a glucose-independent metabolism by reduction of glucose in the culture medium caused tubercidin resistance. High pressure liquid chromatography analysis of glycolytic intermediates from parasites treated with tubercidin showed a dose-dependent increase in concentration of 1,3-bisphosphoglycerate, a substrate of phosphoglycerate kinase. Furthermore, tubercidin triphosphate inhibited recombinant *T. brucei* phosphoglycerate kinase activity *in vitro* with an IC₅₀ of 7.5 μM. We conclude that 5 μM tubercidin kills trypanosomes by targeting glycolysis, especially by inhibition of phosphoglycerate kinase.

*Trypanosoma brucei* is the parasite that causes sleeping sickness, an important disease in Africa. Trypanosomes have complex life cycles with developmental stages in the mammalian host and the tsetse fly vector. *T. brucei* undergoes dramatic changes in metabolism linked to its development. Bloodstream form trypanosomes residing in the mammalian bloodstream lack a Krebs cycle and electron transport chain. They take advantage of the constant supply of blood glucose to generate ATP exclusively through glycolysis. Procyclic form parasites in the tsetse midgut experience a range of nutrient environments and have a more opportunistic metabolism. They utilize glucose or amino acids as carbon and energy sources.

We have described a genomic RNAi library for forward genetic analysis in *T. brucei* (2). For these experiments, we inserted fragments of sheared *T. brucei* DNA into the RNAi vector pZJMβ in which the insert is flanked by T7 promoters under the control of tetracycline operators. We stably integrated this library of genomic fragments into a transgenic procyclic form *T. brucei* cell line that expresses the tet repressor and T7 RNA polymerase (3). Expression of dsRNA from the opposing promoters is induced upon the addition of tet to the culture medium with concomitant degradation of the corresponding mRNA via the endogenous RNAi pathway.

In this study, we were interested in identifying genes whose silencing conferred resistance to the toxic adenosine analog tubercidin (7-deazaadenosine, Tu). Although mutants deficient in adenosine transport in *Leishmania* are resistant to Tu (4), little is known regarding the molecular basis of its toxicity. In mammalian cells, Tu is a substrate for adenosine kinase and becomes incorporated into RNA and DNA (5). In an attempt to discover the target of Tu in *T. brucei*, we screened the RNAi genomic library to select for Tu-resistant cells. To our surprise, we found that silencing of the hexose transporter resulted in Tu resistance. Additionally, RNAi silencing of a glycolytic enzyme or pre-adaptation of parasites to low glucose medium yields Tu-resistant cells. These data suggest that Tu toxicity in trypanosomes is because of its impact on glycolysis, and we have identified phosphoglycerate kinase as a glycolytic enzyme that is inhibited by Tu.

**EXPERIMENTAL PROCEDURES**

*Chemicals—[1,2-3H]2-Deoxyglucose (60 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [3H]Tubercidin (20 Ci/mmol) was from Moravek Biochemicals (Brea, CA). [3H]Adenosine (50 Ci/mmol) was from PerkinElmer Life Sciences. 5′-Tubercidin monophosphate was from Axenza LLC (San Diego, CA). Other chemicals were from Sigma (St. Louis, MO) unless otherwise noted.

*Trypanosoma* Growth and Transfection—Procyclic *T. brucei* 29–13 (a gift from Drs. Elizabeth Wirtz and George Cross), which expresses T7 RNA polymerase and the tet repressor, was grown in SDM-79 supple-

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** The abbreviations used are: RNAi, RNA interference; 1,3-BPG, 1,3-bisphosphoglyceric acid; dsRNA, double-stranded RNA; FGRK, phosphoglycerate kinase B; PKG, phosphoglycerate kinase; tet, tetracycline; THT, trypanosome hexose transporter; Tu, tubercidin; TuMP, tubercidin monophosphate; TuDP, tubercidin diphosphate; TuTP, tubercidin triphosphate; PBS, phosphate-buffered saline; WT, wild type (refers to 29-13 trypanosomes not containing an RNAi insert); HPLC, high pressure liquid chromatography.
mented with 15% fetal bovine serum, G418 (150 μg/ml), and hygromycin (50 μg/ml) (3). For growth in low glucose conditions, trypanosomes were first adapted (for 7 days) to “low glucose medium” (SDM-79 with minimal essential medium replaced by RPMI 1640 medium without glucose (Invitrogen)). This medium also lacked the additional glucose and glucosamine found in standard SDM-79, but it was supplemented with 50 mM proline and 10% fetal bovine serum. The cells were then transferred to “minimal glucose medium” (same as low glucose medium with the exception that dialyzed fetal bovine serum (10%) was used). Transfection of the pZJM library was performed as described previously (2).

**Tubercidin Selection and Analysis of the pZJM Insert**—The adenine-analog tubercidin (7-deazaadenosine) is toxic to procyclic trypanosomes with an EC_{50} (the effective concentration causing 50% reduction in cell viability) of 12 mM (48 h of 0.5 μM) (Fig. 1). To select for Tu-resistant trypanosomes, we induced 50 ml of culture at 1 × 10^{6} cells/ml with 1 μg/ml for 5 days and then added 5 μM Tu to the culture medium. Most cells died within 7 days, and survivors were cloned by limiting dilution in 96-well plates under 5% CO_{2} at 26 °C. Once the cells became visibly turbid, cells were transferred to flask, grown to a density of ~1 × 10^{7} cells/ml, and harvested by centrifugation. Genomic DNA was then isolated using a DNA purification kit (Gentra Systems, Minneapolis, MN), and PCR was performed using primers XlinkF (5'-CTC-GAGGGCCAGTGAGC-3') and XlinkR (5'-AGCTTGAGCCTGAGG-GCC-3') to amplify the unknown fragment within pZJMβ.

The PCR product was cloned into pCR2.1-TOPO (Invitrogen), sequenced and analyzed with BLAST searches. “Re-results,” we found the insert to be a fragment of the trypanosome hexose transporter (ThT) gene. To confirm that RNAi of ThT could confer Tu resistance, we cloned a 350-bp fragment of the open reading frame of ThT (GenBank accession number X86891) by PCR of T. brucei genomic DNA using primers ThT5′ (5'-CACATCACCGGTTTCTTC-3') and ThT3′ (5'-TGGACTTCCCCGGCATAC-3'). This fragment does not overlap with the fragment recovered from the library in Tu-resistant cells and leads to the silencing of both ThT1 and ThT2 family members. The T. brucei hexokinase 1 gene was silenced using a 500-bp fragment of the open reading frame of T. brucei hexokinase 1 gene (GenBank accession number AJ345044). To obtain this fragment, we performed PCR on T. brucei genomic DNA using primers HK2F (5'-CTCTTCCTCCAGGCCATAC-3') and HK2R (5'-GAACGTTACCGGGCATAC-3').

**Kinetic Analysis of Glucose Uptake**—Glucose transport was assayed by a rapid oil-stop method (6). Cells were washed once with PBS and resuspended in PBS to a density of 5 × 10^{6} cells/ml, and 100 μl were layered over 200 μl of assay buffer using a rapid oil-stop method (6). Cells were washed once with PBS and resuspended by vortexing. To begin uptake, 100 μl of assay buffer were added to the Microfuge tube. To stop the reaction, cells were separated from assay buffer by centrifuging (15 min, 3,000 × g). TuTP was purified from the reaction components using an isocratic elution of 50 mM sodium phosphate, pH 7.5, 10 mM NaCl, 50 mM MgSO_{4}, 1 μM of insulin triphosphate, 1 unit of nucleoside monophosphate kinase, 20 units of pyruvate kinase, and 30 nmol of tributylamine, 0.09 M NH_{4}HCO_{3}, 2% CH_{3}CN). TuTP fractions were collected and dried by vacuum drying. The protein was judged to be 99% pure by Coomassie Blue-stained SDS-PAGE.

Coupled PGK activity assays were performed as described previously (11) using a Beckman DU-640 to monitor the change in absorbance at 340 nm. Recombinant PGKβ had a specific activity of 222 units/mg, similar to that observed for recombinant PGKβ lacking a His tag (11). A unit is the amount of enzyme required to convert 1 μmol substrate/mg protein/min. Transfected parasites were pretreated with enzyme for 10 min on ice prior to initiation of the reaction.

**Synthesis and Purification of Tubercidin Triphosphate—TuTP** was synthesized from commercially available TuUMP using a protocol adopted from Schober (12). TuMP (10 μmol) was incubated (15 h, 30 °C) in a 1:1 reaction consisting of 0.2 μM triethanolamine, pH 7.6, 100 mM KCl, 50 mM MgSO_{4}, 1 μM of insomine triphosphate, 1 unit of nucleoside monophosphate kinase, 20 units of pyruvate kinase, and 30 μM of phosphonopyruvate. The pH was adjusted to 7.6 with NaOH. TuTP was purified from the reaction components using an isocratic purification on a C_{18} reverse-phase 2.1 × 150 mm column (25 °C, 125 μl/min, Grace Vydac, Hesperia, CA) using the SMART System (Amer-}
RNA Interference of the Trypanosome Hexose Transporter—THT1 is a member of an 11-gene cluster consisting of six tandem THT1 genes followed by five tandem THT2 genes (the exact copy number depends on the strain) (13). With the exception of ~180 bp, which distinguishes the two families (Fig. 2A, hatched region), these two gene families share a ~80% sequence identity over their coding region (Fig. 2A).

Although we identified a THT1 fragment in our screen, procyclic trypanosomes express predominately THT2 (13). We hypothesized that the high sequence identity between the two genes would result in reduction of mRNA for both types of genes in the pZJM(THT-3') cell line. To test this hypothesis, we
I. Introduction

The glycolytic pathway is a fundamental metabolic process that converts glucose into pyruvate with the production of ATP and NADH. In the case of trypanosomatids, such as Trypanosoma brucei, the glycolytic pathway is crucial for energy production, especially during the procyclic stage when amino acids are the primary carbon and energy source. The inhibition of glycolysis by tubercidin (Tu), a ribose analog of adenosine, can lead to the suppression of trypanosome growth and is a potential target for antiparasitic drugs. However, the exact mechanism of Tu action on the glycolytic pathway is not fully understood.

II. Material and Methods

A. Cell Culture and RNAi

Parental cells were adapted to amino acid metabolism by growth on low glucose medium for 7 days followed by growth in minimal glucose medium. We found that these adapted cells instead on amino acids as a carbon and energy source (14). Parental cells were adapted to amino acid metabolism by growth on low glucose medium for 7 days followed by growth in minimal glucose medium. We found that these adapted cells instead on amino acids as a carbon and energy source (14).

B. HPLC Analysis

Glycolytic intermediates in cell extracts were separated by Dionex chromatography. Inset graphs show enlargements of the region of the 1,3-BPG peak. Labeled peaks were identified by co-migration with standards.

C. Glucose Uptake Assays

To test whether silencing of hexokinase would also confer Tu resistance, we transfected cells with pZJM containing a fragment of the T. brucei hexokinase gene using the construct pZJM(HKORF) (2). Upon induction of RNAi, these cells also became resistant to Tu (EC50 = 10 μM, data not shown), suggesting that a step in glycolysis might be targeted by Tu.

III. Results

A. TuTP inhibits PGKB

Complementary RNA injected

B. 2-deoxyglucose uptake assay

TABLE I

<table>
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<tr>
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<tr>
<td>THT-1</td>
<td>0.225 ± 0.090</td>
<td>0.104 ± 0.022</td>
<td>0.027 ± 0.002</td>
</tr>
<tr>
<td>THT-2</td>
<td>0.298 ± 0.117</td>
<td>0.141 ± 0.052</td>
<td>0.036 ± 0.017</td>
</tr>
<tr>
<td>H2O</td>
<td>0.018 ± 0.005</td>
<td>0.075 ± 0.032</td>
<td>0.026 ± 0.002</td>
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FIG. 3. HPLC of glycolytic intermediates reveals Tu-dependent rise in 1,3-BPG. 29-13 cells were untreated (A) or treated with 25 μM Tu (B) for 60 min. Glycolytic intermediates in cell extracts were separated by Dionex chromatography. Inset graphs show enlargements of the region of the 1,3-BPG peak. Labeled peaks were identified by co-migration with standards.

FIG. 4. TuTP inhibits PGKB. Recombinant PGKB was assayed in the presence of TuTP. Percent inhibition is relative to zero drug. Assays were performed in triplicate.

Therefore, we conducted an experiment based on the finding that procytic trypanosomes can suppress glycolysis and rely instead on amino acids as a carbon and energy source (14). Parental cells were adapted to amino acid metabolism by growth on low glucose medium for 7 days followed by growth in minimal glucose medium. We found that these adapted cells had an EC50 of 5.3 μM, a level of Tu resistance comparable with parental cells (data not shown), suggesting that a step in glycolysis might be targeted by Tu.

Tu Causes an Increase in the Intracellular Concentration of 1,3-Bisphosphoglycerate, a Glycolytic Intermediate—The glycolytic pathway comprises 10 enzymatic steps that convert each molecule of glucose to two molecules of pyruvate with a net production of two ATP molecules. Of these 10 enzymes, 5 bind adenosine in the form of the phosphorylated nucleosides ADP and ATP or as the co-enzymes NAD+ and NADH. We hypothesized that by inhibiting one of these enzymes, Tu, an adenosine analog, might reduce glycolytic flux. We evaluated this possibility by HPLC analysis of the glycolytic intermediates (15). An analysis of extracts from parental cells treated for 60 min with 25 μM Tu resulted in an increase in a peak that elutes...
in a position expected for 1,3-bisphosphoglyceric acid (1,3-BPG) (Fig. 3B). Incubation with 50 and 100 μM Tu confirmed that this effect is dose-dependent (data not shown). This peak is also detected in untreated parasites as a shoulder of a peak co-migrating with fructose 2,6-bisphosphate (Fig. 3A). We identified the 1,3-BPG in two ways. First, using this HPLC chromatography system, 1,3-BPG is known to have a retention time between that of fructose 1,6-bisphosphate and fructose 2,6-bisphosphate (15). The addition of 10 pmol of each of these compounds to the trypanosome extract confirmed that the unknown peak migrates between them (data not shown). Second, we synthesized 1,3-BPG by incubation of glyceraldehyde-3-phosphate with glyceraldehyde-phosphate dehydrogenase, yielding a product with a retention time matching that of the unknown peak (data not shown).

1,3-BPG is a substrate for the seventh enzyme in glycolysis, PGK. The above finding suggested that Tu or a phosphorylated metabolite might cause an increase in the concentration of 1,3-BPG by inhibiting PGK. We found that Tu has no effect on PGK activity in cell extracts, although 1 mM TuMP inhibited activity by 14% (data not shown).

Therefore, we analyzed the impact of Tu nucleotides on purified PGK. *T. brucei* has three PGK genes, *PGKA*, *PGKB*, and *PGKC*, with *PGKB* being the dominant form in procyclic cells (16). We purified recombinant *T. brucei* PGKB and measured the effect of Tu nucleotides on its activity. To assay PGK, we used a coupled reaction that includes glyceraldehyde-3-phosphate dehydrogenase and monitored oxidation of NADH to NAD⁺. We found that TuMP inhibits recombinant PGKB with an IC₅₀ of ~25 μM. AMP also inhibits PGKB with an IC₅₀ ~ 30 μM (data not shown). In a similar coupled reaction using yeast PGK, TuMP and AMP had no effect, indicating that the glyceraldehyde-3-phosphate dehydrogenase in the assay is insensitive to both compounds (data not shown). We then tested the effect of TuTP on recombinant PGKB. This molecule inhibits PGKB with an IC₅₀ ~ 7.5 μM (Fig. 4).

**DISCUSSION**

It seemed likely when we initiated this project that our library selection would reveal that silencing of a nucleoside transporter would lead to Tu resistance as has been found with *Leishmania* Tu-resistant mutants (4). However, since we began these experiments, it was reported that procylic trypanosomes express at least two independent high affinity adenosine transporters (17), a finding that could explain why we did not identify a nucleoside transporter in our selection. Instead, our RNAi library selection has clarified for the first time a mechanism of Tu toxicity in trypanosomes.

Our selection revealed that silencing of a glucose transporter conferred resistance to toxic levels of Tu, suggesting an unexpected link between glucose metabolism and Tu toxicity. Additional experiments confirmed this link. We found Tu resistance in cells with glycolysis suppressed by RNAI silencing of hexokinase 1 or by adaptation to grow in glucose-depleted medium (in the absence of RNAI). How could Tu target glucose metabolism? It has been well established that some adenosine analogs inhibit trypanosome glycolytic enzymes (18). The rationale behind this effect is that all but two of these enzymes contain adenosine-binding domains interacting with either NAD(H) or ADP/ATP. We found that parasites treated with Tu had an increase in the glycolytic intermediate 1,3-BPG, suggesting that Tu or phosphorylated Tu inhibited PGK. Trypanosomes compartmentalize the first seven steps of glycolysis in peroxi-some-like organelles known as glycosomes (19). Bloodstream trypanosomes are completely reliant upon glycolysis for energy with both glycolytic flux and glycosome number being greatly increased. In contrast, procyclic trypanosomes utilize several pathways including glycolysis for energy production. Interestingly, procylic trypanosomes express at least two glycolytic enzymes in the cytosol (1). One of these, PGKB, is the dominant PGK allele in the procyclic stage (20). We found that recombinant PGKB is sensitive to TuMP and TuTP in vitro. We did not test TuDP, which might compete even better with the ADP substrate of PGKB. These data along with the observation that 1,3-BPG accumulates in Tu-treated cells suggest that PGKB is inhibited by phosphorylated forms of Tu. We have not ruled out the possibility that Tu toxicity is the result of elevated levels of 1,3-BPG rather than inhibition of glycolysis.

One paradox is how Tu is toxic to procylic trypanosomes, whereas RNAI silencing of the glucose transporter or growth of these cells in glucose-depleted medium is not. Based on the finding that procylic trypanosomes can shift their metabolism from a state dependent upon glycolysis (in high glucose medium) to one dependent upon amino acids (in low glucose medium) (14), we propose that procylic parasites survive without glycolysis only if they are given time to adapt to a glucose-free environment, a process possibly involving up-regulation of amino acid metabolism or transport. RNAI silencing of the glucose transporter or hexokinase probably occurs over several days, giving time for this adaptation. In contrast, Tu acts quickly, killing the parasites before they can adapt. The fact that depletion of glucose from the culture medium also causes a similar level of Tu resistance (a phenomenon unrelated to RNAI) provides strong evidence that glycolysis is the major target of Tu at the concentrations used in this study.

It is possible that we would identify other genes if we initiated the Tu screen in trypanosomes pre-adapted to amino acid metabolism. Because higher concentrations of Tu are toxic to cells even after adaptation to amino acid metabolism, there must be other pathways also sensitive to Tu.

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