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Short communication

Genetic validation of aldolase and glyceraldehyde-3-phosphate dehydrogenase as drug targets in *Trypanosoma brucei*

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ABSTRACT

Aldolase (ALD) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *Trypanosoma brucei* are considered to be promising targets for chemotherapeutic treatment of African sleeping sickness, because glycolysis is the single source of ATP for the parasite when living in the human bloodstream. Moreover, these enzymes appeared to possess distinct kinetic and structural properties that have already been exploited for the discovery of effective and selective inhibitors with trypanocidal activity. Here we present an experimental, quantitative assessment of the importance of these enzymes for the glycolytic pathway. This was achieved by decreasing the concentrations of ALD and GAPDH by RNA interference. The effects of these knockdowns on parasite growth, levels of various enzymes and transcripts, enzyme activities and glucose consumption were studied. A partial depletion of ALD and GAPDH was already sufficient to rapidly kill the trypanosomes. An effect was also observed on the activity of some other glycolytic enzymes.

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Trypanosoma brucei relies entirely on glycolysis for its ATP supply when living in the bloodstream of humans, inflicting African sleeping sickness upon them. Glucose, abundantly present in the blood, is taken up by the bloodstream-form trypanosomes and, through the enzymes of the glycolytic pathway, converted into pyruvate that is excreted [1]. Because of the parasites' complete dependency on glycolysis, the pathway is considered as a promising target for chemotherapy. There is a desperate need for new effective and safe drugs against sleeping sickness and other human diseases in tropical and subtropical countries caused by related protozoan parasites, because compounds currently used are largely inadequate due to inefficacy, toxicity and increasing drug resistance [2]. Indeed, various glycolytic enzymes have been shown to be essential for the cells by reverse genetic approaches [3,4], or by administration of compounds interfering with glycolysis by inhibition of either the glucose and pyruvate transporters [5,6] or glycolytic enzymes such as aldolase (ALD) [7], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [8,9], phosphoglycerate kinase (PGK) [10], hexokinase (HXK) [11], phosphofructokinase (PFK) and pyruvate kinase (PYK) [12].

The glycolytic pathway in trypanosomatids is organized in a unique way; most enzymes are present in glycosomes, organelles related to the peroxisomes of other eukaryotes [13]. This unusual organisation of the pathway and the long evolutionary distance between trypanosomatids and other organisms have led to the endowment of distinct kinetic, regulatory and structural properties of most glycolytic enzymes. These properties are currently being exploited for the structure-based design of parasite enzyme-selective inhibitors (reviewed in [14]). These approaches have allowed the development of inhibitors for ALD, GAPDH, PFK and PYK that selectively inhibit the parasite enzymes with IC_{50} values in the low or submicromolar range and stunt growth of cultured bloodstream-form trypanosomes while having no effect, or requiring considerably higher doses, on cultured human fibroblasts [8,12,15,16]. Further increase of the potency of these inhibitors may result in lead drugs for human African trypanosomiasis and diseases caused by the related parasites *Trypanosoma cruzi* and various *Leishmania* species.

The importance of some enzymes for the glycolytic pathway has been determined by separately knocking down the concentrations of five glycolytic enzymes by RNA interference (RNAi): HXK, PFK, PYK, PGAM and enolase [4]. These experiments showed that HXK, PFK and PYK are in excess and should be more inhibited than PGAM and enolase to kill the parasites [4]. We decided to extend this study to ALD and GAPDH because structural studies

Abbreviations: ALD, aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPDH, glycerol-3-phosphate dehydrogenase; GPO, glycerol-3-phosphate oxidase; HXK, hexokinase; IC_{50}, inhibitor concentration causing 50% activity decrease; PFK, phosphofructokinase; PGAM, phosphoglycerate mutase; PGK, phosphoglycerate kinase; PYK, pyruvate kinase; RNAi, RNA interference.

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have offered great promise for the design of selective inhibitors of these two enzymes [14]. Indeed, a first generation of such inhibitors has already been developed: adenosine analogues that compete with the cofactor NAD^+ for binding to GAPDH [8,9,17] and compounds that irreversibly bind in the active site of ALD [15]. We present here the quantitative effects of RNAi-dependent depletion of these enzymes on cell growth, enzyme activities and glucose consumption. The effect of RNAi was also tested for other enzymes at both the transcriptional and protein levels.

The knockdown of ALD or GAPDH in bloodstream-form *T. brucei* was accomplished by tetracycline-inducible expression of intramolecular hairpin double-stranded RNA corresponding to either ALD or GAPDH. To produce stem-loop transcripts, constructs were made in pHD677 [18] by cloning, in a direct inverted arrangement, a gene-specific PCR fragment in the sense orientation and the corresponding but somewhat shorter (approximately 50 bp) fragment in the antisense orientation. The constructs were stably integrated in the *T. brucei* genome by transfection as described previously [4].

Growth of wild-type cells, not containing a construct for the production of double-stranded RNA, non-induced cells that contain a construct and cells with a construct induced for RNAi against ALD or GAPDH by the addition of tetracycline were followed during several days. Fig. 1A shows that soon after induction of RNAi for ALD the growth rate was affected, decreasing to 31% during the first 24 h; after 48 h cell growth stopped and the parasites started to die as indicated by the decrease in cell density during the next 36 h. Upon induction of RNAi for GAPDH (Fig. 1B), a decrease of the growth rate to 57% was observed after the first 24 h. The further reduction of GAPDH after 48 h led to growth arrest, followed by trypanosome death. In both cases the effect of the knockdown was lethal for the parasites. No differences were observed between wild-type and non-induced cells (data not shown).

ALD and GAPDH enzyme activities were measured at different times after RNAi induction. In order to determine if the depletion of these proteins had an effect on other enzymes of the glycolytic cascade, other enzymatic activities were also measured. We chose two other glycosomal enzymes, HXK which catalyses the first step of this pathway and PGK-C which functions downstream of the targets and a cytosolically located enzyme, PYK. Fig. 2A shows a selective and quick decrease of the ALD activity to $27 \pm 3.9\%$ of that of non-induced cells during the first 24 h of induction of RNAi for ALD. During the same time the activities of HXK and PGK-C remained constant, but a small effect was observed on the PYK activity, which dropped to $72.7 \pm 1.2\%$. When the period of induction of RNAi for ALD was extended to 72 h, the enzyme activity decreased gradually to $5.3 \pm 2.9\%$. Concomitantly the activities of the PGK-C and PYK dropped sharply to $58.7 \pm 9\%$ and $20.0 \pm 0.27\%$, respectively. An increase was observed for the HXK activity (to $115.5 \pm 5.5\%$), similar to the result that was obtained previously when PYK was targeted by RNAi [4]. Upon induction of RNAi for GAPDH (Fig. 2B), its activity gradually decreased during the first 36 h until $72.2 \pm 8.1\%$ of that of non-induced cells, with no significant changes of the HXK and PGK-C activities. The PYK activity showed a decrease to $77.9 \pm 3.2\%$ as was observed when the ALD was targeted by RNAi. Between 36 and 48 h after induction, the activities of GAPDH and PYK continued to drop (to $46.3 \pm 6.2\%$ and $38.4 \pm 8.2\%$, respectively), but also, to a lesser extent, the HXK activity to reach $78.5 \pm 3.2\%$ after 48 h of induction. The activity of PGK-C remained essentially unchanged.

The efficiency of mRNA depletion by RNAi was checked by semi-quantitative RT-PCR using total RNA extracts prepared from cells sampled at different times of induction of RNAi. The levels of mRNA for glycolytic enzymes other than ALD and GAPDH were also measured; the values were normalized to the tubulin

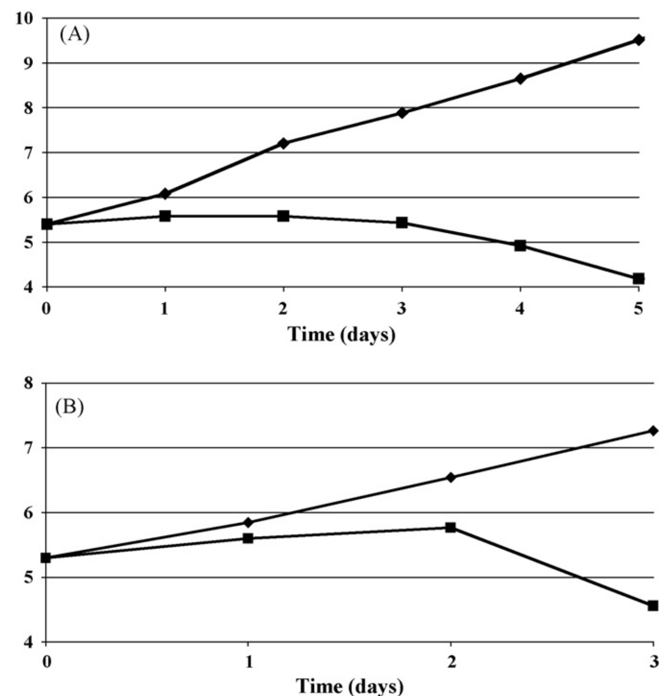

Fig. 1. Effect of intracellular depletion of ALD (panel A) and GAPDH (panel B) by RNAi on the growth of bloodstream-form *T. brucei*. To express, under tetracycline (Tet) induction, an intramolecular ('hairpin') double-stranded RNA able to trigger RNAi against ALD or GAPDH in trypanosomes, constructs were prepared by cloning, in an inverted arrangement, a gene-specific PCR fragment in the sense orientation and the corresponding but somewhat shorter fragment in the antisense orientation in plasmid pHD677 [18]. The sequence of the *T. brucei ALD* locus was retrieved from the NCBI GenBank (accession no. X52587). Specific primers were designed in order to amplify 452 bp of the 3′UTR flanking the *ALD* genes: for the amplification of the 3′UTR in the sense orientation, the oligonucleotide 5′-CCG**AAGCTT**GCCCATTTAGTTGGCTTTTCCCT-3′ (corresponding to bp 10–22 downstream of the gene stop codon; HindIII site in bold) was used as the forward primer and 5′-GCC**CCATGG**TATATTCCTCTCATACACACAAAAG-3′ (3′UTR bp 438–462; NcoI site in bold) as the reverse primer; for the amplification of the fragment in the antisense orientation, the forward primer was 5′-CCG**CCATGG**TTCGTTTTTTACCACTTTTCAAAAGG-3′ (3′UTR bp 385–410; NcoI site in bold) and the reverse primer 5′-GCC**GGATCC**GCCCATTTAGTTGGCTTTTCCC-3′ (3′UTR bp 10–21; BamHI site in bold). For GAPDH (GenBank accession no. X59955) the specific primers designed to amplify a 404 bp fragment of the 3′UTR were the following: for the amplification of the 3′UTR in the sense orientation, as the forward primer 5′-CCG**AAGCTT**CTACGCGCTTGGCTGTGGTGCG-3′ (corresponding to bp 17–38 downstream of the gene stop codon; HindIII site in bold) and the reverse primer 5′-GCC**CCATGG**TAAACCTTGCTCAATTATGCATCCATTTCC-3′ (3′UTR bp 392–421; NcoI site in bold); for the amplification of the fragment in the antisense orientation, the forward primer used was 5′-CCG**CCATGG**CACAATTCATATATTTTCCCCAATTTTC-3′ (3′UTR3′ bp 337–364; NcoI site in bold) and the reverse primer 5′-GCC**GGATCC**CTACGCGCTTGGCTGTGGTGCG-3′ (3′UTR bp 17–38; BamHI site in bold). The amplified products were first cloned in vector pTZ57R (Fermentas) and then transferred to vector pHD677, downstream of the Tet-inducible PARP promoter. The recombinant plasmids pHD677-ALD and pHD677-GAP were linearized to accomplish their integration in the ribosomal repeat spacer of the *T. brucei* genome as described in [4]. Bloodstream-form *T. brucei* 427, cell line 449 [18] constitutively expressing the *Tn10* tetracycline (*Tet*) repressor, was grown as described before [4] in HMI-9 medium containing 10% of heat-inactivated fetal bovine serum (Gibco) and 0.2 μg/ml phleomycin (Sigma), the selectable marker for the *Tet* repressor construct, at 37 °C under water-saturated air with 5% CO_2. For induction of double-stranded RNA, Tet was added at a concentration of 1 μg/ml. Cultures of 1 ml were diluted daily to 2.10^5 cells/ml in 24-well plates. Cell densities were determined using a cell counting grid (Bürker-Türk with a depth of 0.01 mm) and growth curves were plotted as the product of cell density and total dilution versus time. The growth of transfected trypanosomes was determined for cells cultured in the absence (♦) or the presence (■) of Tet and compared with the growth of cell line 449 (not shown). For each construct, a single clone was selected for further analysis. The growth curve was repeated for this clone.

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