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Inhibition of *Trypanosoma brucei* glucose-6-phosphate dehydrogenase by human steroids and their effects on the viability of cultured parasites

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ABSTRACT

Dehydroepiandrosterone (DHEA) is known as an intermediate in the synthesis of mammalian steroids and a potent uncompetitive inhibitor of mammalian glucose-6-phosphate dehydrogenase (G6PDH), but not the enzyme from plants and lower eukaryotes. G6PDH catalyzes the first step of the pentose-phosphate pathway supplying cells with ribose 5-phosphate, a precursor of nucleic acid synthesis, and NADPH for biosynthetic processes and protection against oxidative stress. In this paper we demonstrate that also G6PDH of the protozoan parasite *Trypanosoma brucei* is uncompetitively inhibited by DHEA and epiandrosterone (EA), with K_i values in the lower micromolar range. A viability assay confirmed the toxic effect of both steroids on cultured *T. brucei* bloodstream form cells. Additionally, RNAi mediated reduction of the G6PDH level in *T. brucei* bloodstream forms validated this enzyme as a drug target against Human African Trypanosomiasis. Together these findings show that inhibition of G6PDH by DHEA derivatives may lead to the development of a new class of anti-trypanosomatid compounds.

1. Introduction

Trypanosoma brucei, *Trypanosoma cruzi* and *Leishmania* spp. are parasitic protozoans responsible for diseases that affect mankind in tropical and subtropical countries: Human African Trypanosomiasis or sleeping sickness, Chagas disease and leishmaniasis, respectively. Control of such diseases relies mainly on chemotherapy, but current drugs are highly inadequate. Recently, most efforts have been directed to the development of drugs that act specifically on validated metabolic targets. Several enzymes of carbohydrate metabolism, participating in glycolysis[1] and the pentose-phosphate pathway[2] (PPP), have been perceived as promising targets for drug design strategies. The use of RNA interference (RNAi) in *T. brucei* has become the preferable approach to identify essential proteins for growth and survival of the parasites. Expression of double-stranded RNA to induce RNAi against hexokinase, phosphofructokinase, pyruvate kinase, phosphoglycerate mutase and enolase was used to evaluate the importance of these enzymes for the glycolytic flux and growth of cultured pathogenic bloodstream-stage forms of the trypanosome.[3]

Glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the first step of the PPP, converting glucose 6-phosphate (G6P) into 6-phospho-gluconolactone and reducing $NADP^+$ to NADPH. The oxidative branch of the PPP produces ribose 5-phosphate (R5P) which is a precursor for nucleic acid synthesis and supplies the cell with NADPH for biosynthetic processes and protection against oxidative stress. G6PDH deficiency affects 400 millions of people in the world, it is the most common human enzyme pathology. Most G6PDH-deficient individuals are asymptomatic and unaware of their status. The illness generally manifests itself as acute haemolysis, trigged by oxidative stress conditions.[4] G6PDH deleted embryonic stem cells can be cultured in usual Dulbecco's Modified Eagle's medium, although with an impaired cloning efficiency; that can be overcome by reducing the oxygen pressure of the medium.[5] In such cells, the NADPH production is maintained by cytosolic isocitrate dehydrogenase and malic enzyme; G6PDH is overexpressed in response to oxidative agents.[6] In trypanosomatids, isocitrate dehydrogenase is a mitochondrial enzyme and in *T. cruzi* and *T. brucei*, but not *Leishmania* species, it is also predicted to occur inside the peroxisome-like organelles called glycosomes.[7] Therefore, trypanosomatids rely on malic enzyme[8] and both dehydrogenases of the PPP (G6PDH and 6-phospho-gluconolactone dehydrogenase, 6PGDH) for cytosolic NADPH production.

The possibility of using the PPP of *T. brucei* as a drug target is supported by studies on 6PGDH. Differences between sheep and *T. brucei* 6PGDHs structures[9] permitted the design of selective inhibitors with trypanocidal activity.[10] It is noteworthy that 6PGDH inhibition leads to accumulation of 6-phospho-gluconate, which is a competitive inhibitor of glucose-6-phosphate isomerase in glycolysis.[11]

Dehydroepiandrosterone (DHEA) is an intermediate in the synthesis of mammalian steroid hormones such as testosterone and estradiol. It is produced in the adrenal cortex and reaches maximal

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values in the circulation between the ages of 20 and 30 years; thereafter, the serum DHEA level decreases markedly.[12] Its administration to elderly people has been associated to ameliorating the signs of the aging process and some derivatives have been synthesized aiming the development of new drugs against cancer, diabetes, obesity and auto-immune diseases.[13] DHEA affects mammalian cell proliferation, but the precise mechanism by which it does so is unclear. The enzymes G6PDH of the PPP and 3-hydroxy-3-methyl glutarylCoA reductase (HMGR) of the mevalonate pathway are inhibited by DHEA. HMGR catalyzes the rate-limiting step in sterol biosynthesis and its product, mevalonate, is a precursor of farnesyl diphosphate, necessary for protein farnesylation reactions occurring in many cellular processes such as signal transduction.[14] One observation in favor of G6PDH as the main target is that cell growth suppression by DHEA was partially prevented by addition of ribonucleosides, but not mevalonolactone.[15] Probably, the growth inhibition effect of DHEA might not be attributed to a single mechanism, but G6PDH inhibition seems to be an important contributing factor, either by reduction of 5-carbon sugars for the synthesis of ribonucleosides and deoxyribonucleosides or by depletion of NADPH, the reductive agent for lipid and cholesterol biosynthesis and reactive oxygen species (ROS) detoxification.[16]

DHEA is an uncompetitive inhibitor of mammalian G6PDH, but not the enzyme from plants and lower eukaryotes.[17,18] Nevertheless, DHEA derivatives were effective against parasitic infections by *Plasmodium falciparum*,[19] *Cryptosporidium parvum*,[20] *Schistosoma mansoni*,[21] and *T. cruzi*.[22] In such cases, it was suggested that DHEA acts by stimulating the host immune response. In the case of parasitic infections by *Taenia crassiceps*[23] and *Entamoeba histolytica*,[24] DHEA presented a parasiticidal effect not related to the immune response. The anti-parasitic properties of DHEA are remarkable, but more information is needed to clarify its mechanism of action.

In the work presented here, we (1) evaluate the effect of depleting G6PDH transcript levels in *T. brucei* bloodstream form by RNAi; (2) determine kinetic constants for recombinant *T. brucei* and *Leishmania mexicana* G6PDH (*Tb* and *Lm*G6PDH), demonstrating that both enzymes form ternary complexes with their substrates G6P and $NADP^+$; (3) measure DHEA and EA inhibition constants against *Tb*G6PDH and (4) assay the effect of DHEA and EA on growth and viability of cultured *T. brucei* and *L. mexicana*. All together, the results indicate that DHEA (or EA) might be explored as a template molecule for the development of new anti-trypanosomatid compounds that act specifically against the parasites' G6PDH.

2. Results

2.1. Conditional depletion of G6PDH levels by RNAi

To genetically validate G6PDH as a drug target in bloodstream-form *T. brucei*, a cell line was created by transfection with a construct enabling the production of double-stranded RNA corresponding to the G6PDH gene from a tetracycline-inducible promoter, and so to specifically decrease the level of the enzyme by RNAi. A reduction in the growth rate of bloodstream-form cells was observed between 24 and 48 h after tetracycline addition to the culture. After 48 h, *Tb*G6PDH depleted cells began to die. Non-induced transfected trypanosomes presented a similar growth rate as wild-type cells cultured in the presence or absence of tetracycline (Fig. 1A). No decrease of intracellular *Tb*G6PDH levels was observed after 24 h of RNAi induction, but at 48 h, the level of *Tb*G6PDH in cellular extract was considerably reduced, almost undetectable by western-blot analysis (Fig. 1B). The reduction of the growth rate of the culture thus correlates with the beginning of the RNAi induced decrease of the protein level, indicating that

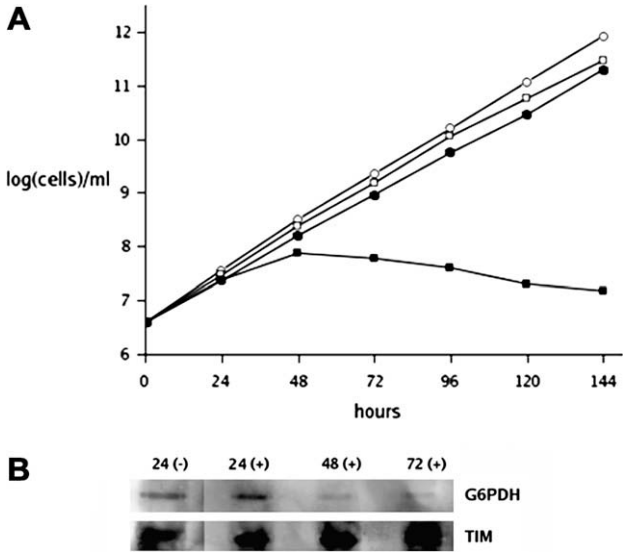

Figure 1. (A) Cumulative growth curve of *T. brucei* bloodstream forms transfected with a G6PDH RNAi construct. In the absence of RNAi induction, both wild-type (○) and transfected (□) cells grow at equal rates. In the presence of the RNAi-inducer tetracycline, wild-type cells (●) grow at normal rates, while transfected cells (■) die after 48 h. (B) Western blot analysis of the G6PDH levels in *T. brucei* bloodstream form cells transfected with a RNAi construct. Samples were obtained at 24 h intervals from cultures grown in the absence (−) and presence (+) of tetracycline. Triosephosphate isomerase (TIM) was used as a sample loading control.

*Tb*G6PDH exerts control on the growth or viability of the bloodstream-form trypanosomes. The possibility that levels of other, unknown essential proteins are affected by off-target effects and contribute to the observed growth phenotype cannot be excluded but is considered unlikely. The G6PDH sequence is unique; no other genes with stretches of DNA highly similar to that of the G6PDH gene were detected in the *T. brucei* genome database.

2.2. *T. brucei* and *L. mexicana* G6PDH kinetic mechanism

N-terminally His-tagged recombinant *Tb*- and *Lm*G6PDH were expressed in *Escherichia coli* BL21 transformed with recombinant pET28 plasmids containing either of the parasites genes, and purified to homogeneity by a single nickel affinity chromatographic step. Approximately 1 and 3 mg of essentially pure *Tb* and *Lm*G6PDH were recovered per liter of *E. coli* culture. Enzyme kinetics analyses were performed for both His-tagged recombinant G6PDHs. Reaction velocities, expressed as nmoles of NADPH produced per second, were obtained by varying the G6P concentration at different concentrations of $NADP^+$ (Fig. 2). The convergent patterns of Lineweaver-Burk lines observed for both *Tb* and *Lm*G6PDH are consistent with a bi-bi ordered reaction mechanism.[25] Linear fit to secondary plots ($1/V_{max}^{app\text{-}g6p}$ vs $1/[NADP^+]$ and $(K_m/V_{max})^{app\text{-}g6p}$ vs $1/[NADP^+]$) allowed to calculate kinetic constants using Cleland's velocity equation:

$$v = V_1[A][B]/(K_{ia}K_b + K_b[A] + K_a[B] + [A][B]),$$

where K_a and K_b are the Michaelis constants for substrates A (G6P) and B ($NADP^+$), respectively; and V_1 and K_{ia} are the maximum velocity and the dissociation constant for substrate A, respectively. The kinetic constants derived for *Tb* and *Lm*G6PDH are presented in Table 1.

2.3. Inhibition of G6PDH by DHEA and EA

DHEA and EA are uncompetitive inhibitors with respect to both substrates, G6P and $NADP^+$, of *Tb*G6PDH, but not in the case of

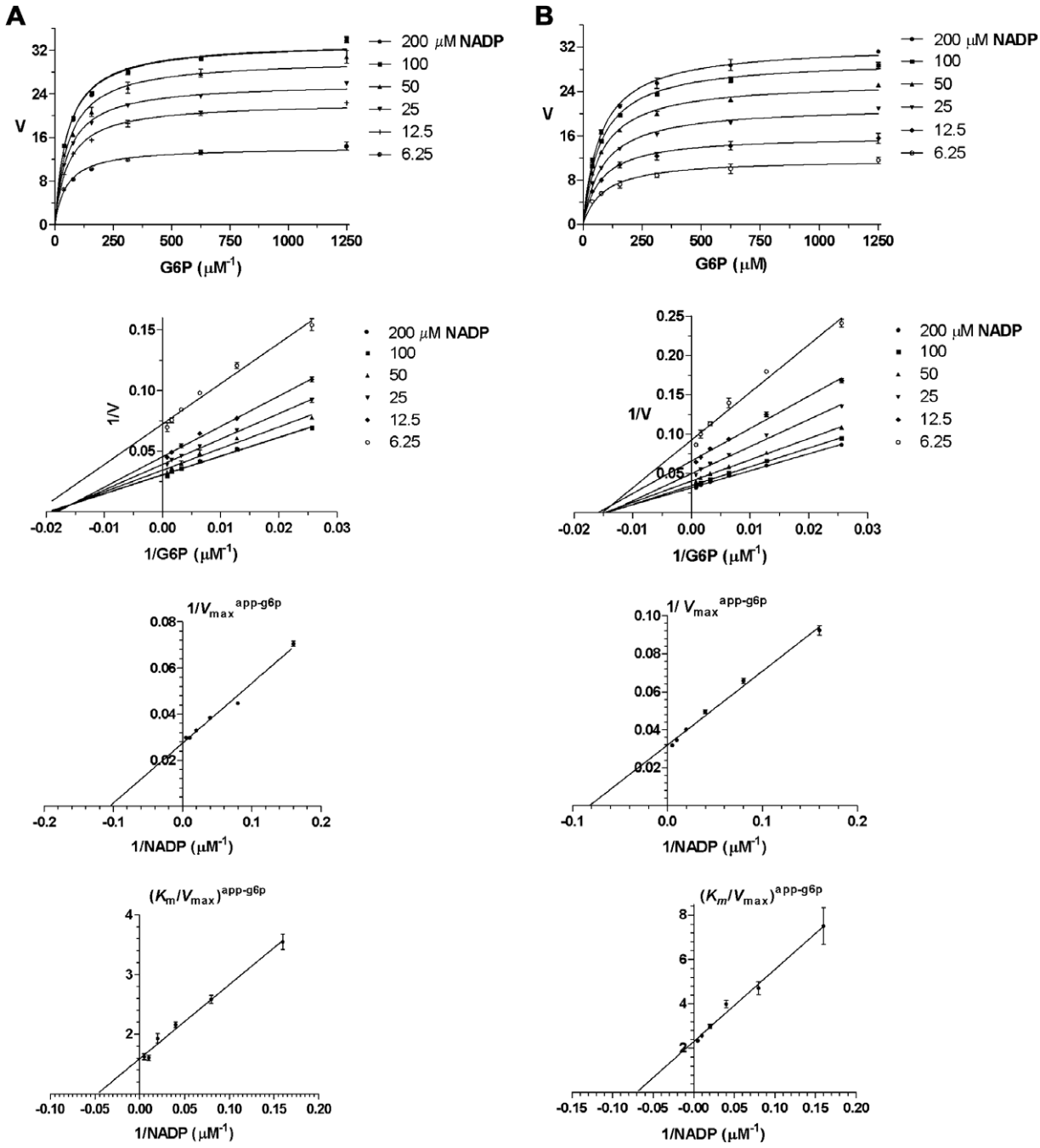

Figure 2. Reaction velocity, Lineweaver-Burk and secondary plots for *Tb* (A) and *Lm*G6PDH (B). Velocity (V) is expressed in nmoles of NADPH produced per second. In velocity and Lineweaver-Burk plots, each curve was obtained at a fixed concentration of $NADP^+$ (as indicated aside of the plots) while the G6P concentration was varied (at values of 1250, 625, 312.5, 156.25, 78.12 and 39.06 μM).

Table 1
T. brucei (Tb), and *L. mexicana* (Lm) G6PDH kinetic constants for an ordered bi-bi reaction mechanism

G6PDH	K_{G6P} (μM)	K_{NADP} (μM)	V_1 (nmoles of NADPH s^{-1})	k_{cat} (s^{-1})	K_{iG6P} (μM)
Tb	57.8 ± 2.4	9.4 ± 0.4	36.2 ± 1.5	16.4 ± 0.6	47.6 ± 1.9
Lm	74.5 ± 3.0	12.1 ± 0.5	31.2 ± 1.2	22.2 ± 0.9	86.4 ± 3.5

*Lm*G6PDH. The uncompetitive inhibition is characterized by proportional changes in apparent K_m and V_{max}, which result in parallel Lineweaver-Burk lines for different inhibitor concentrations (Fig. 3A and B). Due to solubility limitation, the highest EA and DHEA concentrations achieved in *Lm*G6PDH inhibition assays were 50 and 100 μM, respectively. At these concentrations, the compounds had no significant effect on *Lm*G6PDH, resulting in Lineweaver-Burk lines that superpose to those obtained in the inhibitor-free assay (Fig. 3C). EA and DHEA K_i values were calculated for each of the *Tb*G6PDH substrates separately (Table 2).

2.4. *T. brucei* and *L. mexicana* viability assays

The reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) to formazan was used as a measure of viable cells grown

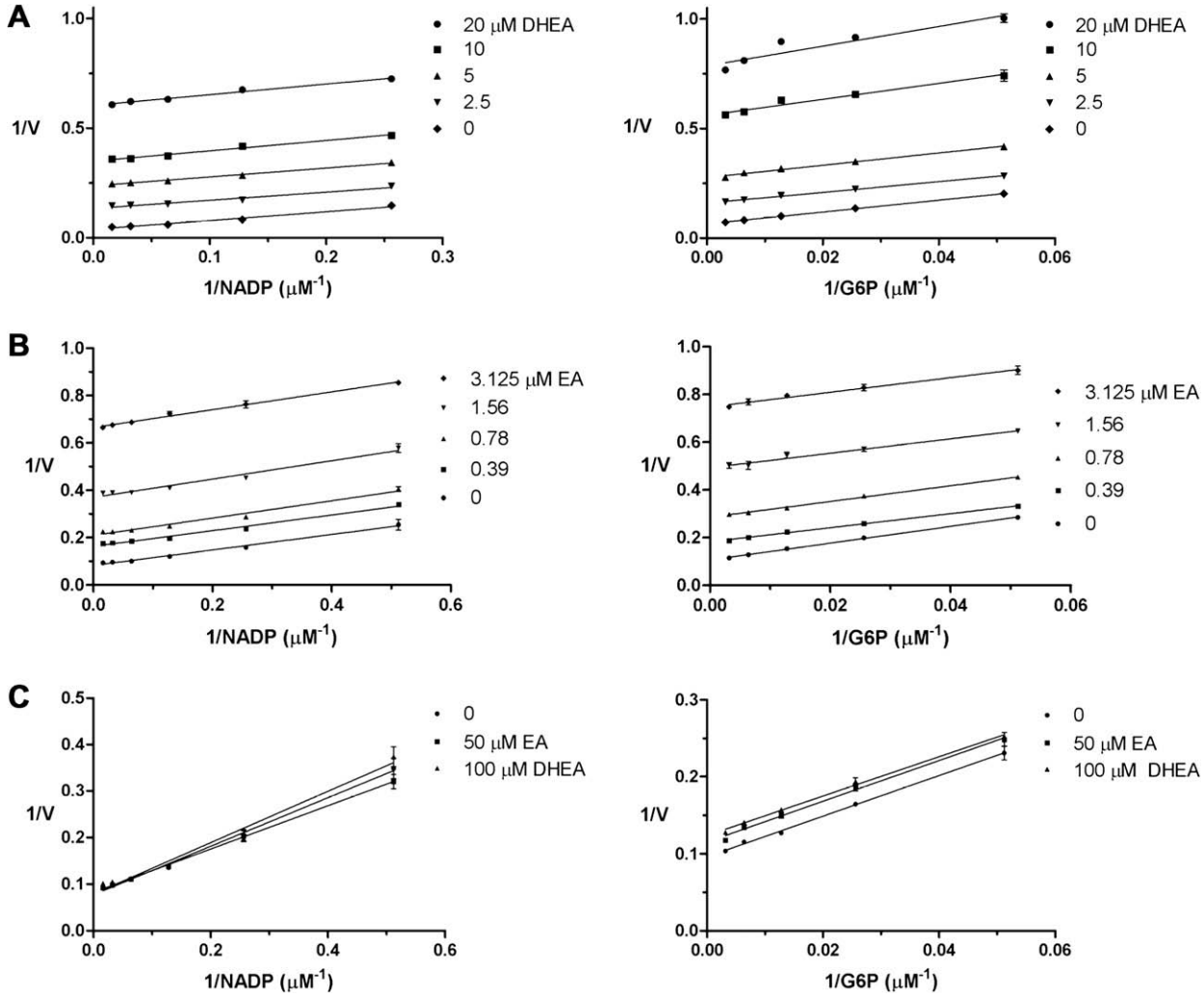

Figure 3. Dehydroepiandrosterone (DHEA) and Epiandrosterone (EA) inhibition of *T. brucei* (A and B) and *L. mexicana* G6PDH (C). Inhibitors were assayed against each substrate in separate experiments. First, G6P was varied from 312.5 to 19.5 μM with $NADP^+$ fixed at a saturating concentration (250 μM), then $NADP^+$ was varied from 31.25 to 1.95 μM while G6P was kept at a saturating concentration (1.25 mM). The parallel lines in the Lineweaver-Burk plots confirm that DHEA is an uncompetitive inhibitor of *Tb*G6PDH.

Table 2
Comparison of inhibition constants of Dehydroepiandrosterone (DHEA) and Epiandrosterone (EA) between *T. brucei* (*Tb*G6PDH) and human (*Hs*G6PDH) glucose-6-phosphate dehydrogenase

	*Tb*G6PDH		*Hs*G6PDH[a]	
	G6P	NADP	G6P	NADP
$K'_{i\,DHEA}$ (μM)	1.70 ± 0.1	1.10 ± 0.1	8.9 ± 0.3	6.2 ± 0.5
$K'_{i\,EA}$ (μM)	0.49 ± 0.03	0.46 ± 0.03	3.4 ± 0.3	3.0 ± 0.1

[a] Values extracted from Gordon et al., 1995.[18]

in the presence of different concentrations of DHEA and EA. LD_{50} was defined as the inhibitor concentration that reduces the number of viable cells by 50%. The LD_{50} of EA (LD_{50}^{EA}) and DHEA (LD_{50}^{DHEA}) for *T. brucei* bloodstream forms were 24.5 ± 0.7 and 43.8 ± 2 μM, respectively. The EA and DHEA toxicity data for *T. brucei* fitted a dose–response curve with R^2 = 0.995 and 0.984, respectively (Fig. 4A). *L. mexicana* was not affected by the presence of EA and DHEA in the culture medium (Fig. 4B). At concentrations superior to 96 μM, crystalline precipitates were observed in the *L. mexicana* assays hampering the measurements at higher concentrations.

3. Discussion

3.1. *T. brucei* G6PDH is a valid drug target

The conditional RNAi-dependent G6PDH depletion in transfected *T. brucei* bloodstream cells causes a reduction in growth rate followed by culture death after 48 h (Fig. 1). This result strongly suggests that *Tb*G6PDH is an essential enzyme for proliferation of *T. brucei* bloodstream forms and a valid drug target against Human African Trypanosomiasis.

NADPH, more than R5P, is pointed as the most important end product of the oxidative branch of the PPP to sustain mammalian embryonic stem cells cloning efficiency.[5] In G6PDH deficient cells, R5P might be provided by the successive action of transaldolase and transketolase on fructose 6-phosphate, an intermediate of glycolysis. In trypanosomatids, the first seven glycolytic steps occur inside a peroxisome-like organelle denominated glycosome; other pathways like the PPP, β-oxidation of fatty acids, purine salvage, and biosynthetic pathways for pyrimidines, ether-lipids and squalenes are also at least partially localized inside glycosomes.[26] In *T. brucei* procyclic forms, G6PDH is found in both glycosomes and cytosol;[27] this dual subcellular localization is evidence that NADPH is required inside glycosomes too for the biosynthetic processes

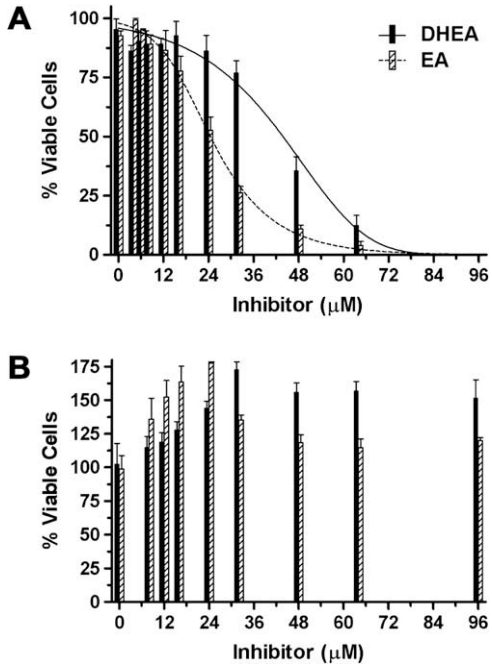

Figure 4. *T. brucei* bloodstream forms (A) and *L. mexicana* early-metacyclic promastigotes (B) viability at different concentrations of DHEA and EA. Dose–response curves for DHEA and EA are presented in the *T. brucei* plot.

located in them, and also because a cascade of detoxification reactions is initiated in these organelles with the reduction of trypanothione by a NADPH-dependent trypanothione reductase, an enzyme that also has been shown to have a dual localization.[28]

3.2. Inhibition of *T. brucei* G6PDH by human steroids

EA and DHEA are potent inhibitors of *Tb*G6PDH, with K_i' values in the low micromolar range. An initial study on the interaction of G6PDH with human steroids detected an inhibitory effect on purified human enzyme and the enzyme in rat crude tissue extracts but not in spinach extracts or on partially purified yeast enzyme.[17] Based on these findings it was suggested that only mammalian G6PDHs were inhibited by DHEA derivatives.[18] However, we found that *Tb*G6PDH is also inhibited by DHEA and EA, and that the parasite's enzyme has even a higher affinity (lower K_i) than does *Hs*G6PDH. Comparison of DHEA and EA effects on *Tb* and *Hs*G6PDH (Table 2) shows that inhibition constants for the protozoan enzyme are approximately 6-fold lower than the values obtained for the human enzyme. To our knowledge, *Tb*G6PDH is the first non-mammalian enzyme for which uncompetitive inhibition by human steroids has been demonstrated. Uncompetitive inhibition is rarely observed in known cell metabolism. The specific interaction of an uncompetitive inhibitor with an enzyme–substrate ternary complex can increase metabolic intermediates to toxic concentrations inside cells.[29] The formation of a ternary complex is essential for the binding of uncompetitive inhibitors. Here, we demonstrate that *Tb* and *Lm*G6PDH follow the ordered bi-bi reaction mechanism, in which both substrates bind the enzyme, forming a ternary complex, before the chemical reaction occurs. At this stage no information is available about the localization of the steroid binding site in the trypanosomatid G6PDH. Crystallographic structures for NADP-dependent G6PDHs are known for only two species: *human*[30,31] and the bacterium *Leuconostoc mesenteroides*.[32,33] The latter is not inhibited by DHEA and the structure of the human enzyme did not reveal a possible binding site to DHEA.

3.3. DHEA and EA are toxic to *T. brucei*, but not to *L. mexicana*

The observation that DHEA and EA are toxic for in vitro cultures of *T. brucei* bloodstream forms but not *L. mexicana* early-metacyclic promastigotes is in agreement with the notion that they arrest parasite growth by specifically inhibiting G6PDH. Our results clearly show that EA and DHEA are potent inhibitors of only *Tb*G6PDH, but not of *Lm*G6PDH and they also confirm that both steroids cause death of trypanosomes, but do not affect *Leishmania* growth. Although off-target effects, like HMGR inhibition, remain to be excluded, our data suggest that EA and DHEA toxicity for trypanosomes is most likely a consequence of G6PDH inhibition. If other targets than G6PDH exist in *T. brucei* bloodstream form, their homologues in *L. mexicana* must also be unsusceptible to DHEA, otherwise we would expect a *L. mexicana* culture to be killed once challenged with DHEA or EA. It may be possible that DHEA also interferes with other vital cellular processes; however, the inhibition of G6PDH certainly impairs *T. brucei* bloodstream forms proliferation, as observed by RNAi experiments targeted against *Tb*G6PDH.

It is also worth noting that in *T. brucei* bloodstream form, differently from other trypanosomatids, cholesterol is the predominant sterol and it is scavenged from host serum lipoproteins; *Leishmania* species synthesize predominantly ergosterol-related molecules, although in its amastigote form it can also acquire cholesterol from the host macrophage.[34] The capability of surviving with a variable profile of sterol may assist the parasites to overcome an inhibition of sterol biosynthesis and it imposes additional challenges to the development of specific inhibitors for such pathway enzymes.

4. Conclusion

Contrary to the general concept that DHEA and its derivatives are specific inhibitors of mammalian G6PDHs, we showed here that DHEA and EA are also potent inhibitors of the G6PDH of the protozoan *T. brucei*. The protective effect of DHEA derivatives on different parasitic infections has previously been attributed to the enhancement of the host immune response. However, we demonstrated that DHEA derivatives may also be cytotoxic against human parasites such as *T. brucei* bloodstream forms. Inhibition of parasite G6PDH, if not lethal by interrupting an essential glucose-catabolizing process, may at least decrease the parasite's tolerance to the oxidative burst mediated by host defensive cells. Based on the presented results, DHEA derivatives may thus be explored as a novel class of anti-trypanosomal agents due to their inhibitory effect on the G6PDH. In order to be considered efficacious anti-trypanosomal molecules, selectivity against the parasite enzyme and minimal androgenic effect are desired properties for DHEA derivatives to be developed. We are currently conducing crystallization trials to determine the structure of *Tb*G6PDH in complex with EA. Information about such a complex will help us in reaching the desired selective anti-trypanosomal properties for DHEA derivatives.

5. Experimental

5.1. RNA interference

Conditional depletion of *Tb*G6PDH by interfering with its expression was performed similarly as in the previously reported RNAi experiments for *T. brucei* glycolytic enzymes.[3] To induce RNAi in *T. brucei* bloodstream forms, a construct was prepared for the tetracycline-inducible synthesis of a double-stranded RNA hairpin molecule. The construct consists of two short, complementary DNA fragments of the *Tb*G6PDH gene (Tb10.70.5200, 1677 bp), one

somewhat longer than the other, that were inserted side by side but in opposing directions into vector pHD677,[35] and so allowing the synthesis of a transcript with a stem-loop structure. The primers used in PCR were Z1, 5′-AAAGCTTATTCGTGGTACTTCGAC-3′ (*Hind*III site underlined); Z2, 5′-AGGGCCCGCTGCTGGTAAATATCC-3′ (*Apa*I site underlined); Z3, 5′-AGGATCCATTCGTGGTACTTCGAC-3′ (*Bam*HI site underlined) and Z4, 5′-AGGGCCCTCATCAACAAACTTTTG-3′ (*Apa*I site underlined). The sense-loop (521 bp) and anti-sense (494 bp) fragments were amplified from the 3′-terminal gene region, using primer pairs Z1/Z2 and Z3/Z4, respectively. The PCR products have been cloned first in pCR2.1-TOPO, sequenced and subsequently simultaneously ligated into pHD677 between the *Hind*III and *Bam*HI restriction sites. The recombinant plasmids have been screened by double digestion with *Hind*III and *Bam*HI (insert 1029 bp) and by a single digestion with *Apa*I (approximately 7 kb).

Prior to transfection of *T. brucei* bloodstream forms, the pHD677-G6PDH construct was linearized by *Not*I digestion to permit its homologous recombination into a transcriptionally silent region of the genome, the spacer of the ribosomal RNA gene repeat. For transfection, 1×10^7 *T. brucei* bloodstream cells, grown in HMI-9 medium supplemented with 10% Fetal Bovine Serum (FBS), were harvested by centrifugation, washed twice in Cytomix solution and resuspended in 1 ml of the same solution. 0.4 ml of Cytomix solution containing *T. brucei* bloodstream cells was incubated with 10 μg of the *Not*I linearized DNA construct and submitted to an electric discharge from a Genetronics BTX ECM630 electroporator set to 1250 V, 25 Ω, and 50 μF to obtain a pulse with a voltage of 800 V and a time constant of 275 μs. After the electric discharge cells were transferred to 12 ml of HMI-9 medium (10% FBS) and incubated at 37 °C (5% CO_2). After 24 h, hygromycin was added to the medium and the culture split in a 24 wells microtiter plate. Antibiotic resistant cells were detectable after 7–10 days.

5.2. *Tb* and *Lm*G6PDH expression and purification

The cloning of the *Tb*G6PDH gene in the pET28 expression vector has been described elsewhere.[36] The *Tb*G6PDH gene was used as a probe for identifying the *Lm*G6PDH gene in a genomic library of *L. mexicana* strain NHOM/B2/84/BEL46.[37] The *Lm*G6PDH gene was subcloned in the pET28a vector between the *Nde*I and *Bam*HI restriction sites. *Lm* and *Tb*G6PDH were overexpressed in *E. coli* BL21(DE3) transformed with the recombinant pET28 constructs and incubated in ZYM-5052 autoinduction medium for 48 h at 25 °C. Cells were harvested by centrifugation at 4000*g* during 20 min and disrupted in a French Press cell at 13,000 psi. His-tagged *Lm* and *Tb*G6PDH were purified to homogeneity by metal affinity chromatography on columns containing 2 ml HisLink resin (Promega) following the manufacturer's recommended procedure. *Lm* and *Tb*G6PDH were eluted in 50 mM tricine pH 8, 0.5 M NaCl and 0.4 M imidazole.

5.3. Measurements of steady-state kinetic parameters

In order to determine the reaction mechanism of *Tb* and *Lm*G6PDHs, the rates of NADPH formation were measured at 340 nm in a SpectraMax Plus^{384} spectrophotometer (Molecular Devices), using a 96 well titer plate and a final reaction volume of 0.2 ml per well. All pipetting steps were performed automatically in a Biomek3000 robot (Beckman Coulter). Initially, 5 μl of eight different G6P stock solutions were loaded into the wells of the first four columns. The reaction was started simultaneously in all wells of each column by addition of a mix solution containing the enzyme (2.2 nM of *Lm*G6PDH or 1.4 nM *Tb*G6PDH) and $NADP^+$ (200, 100, 50, 25 or 12.5 μM) in *RB* reaction buffer (50 mM triethanolamine pH 7.6, 2.5 mM $MgCl_2$). The robot took less then 20 s to load solutions into the four columns of the plate. The concentration of *Lm* or *Tb*G6PDH in the mix solution was chosen in order to allow the measurement of constant NADPH formation rates for a minimum of 60 s. In wells from each plate's line the reaction occurs at different G6P (1250, 625, 312.5, 156.25, 78.12, 39.06, 19.53 and 9.77 μM) and a unique $NADP^+$ concentration. The procedure was repeated for the different $NADP^+$ concentrations. From the program GraphPad Prism v.5.0 (GraphPad), a non-linear fit module was used to calculate $K_m^{app\text{-}g6p}$ and $V_{max}^{app\text{-}g6p}$ for different $NADP^+$ concentrations, while a linear regression module transformed the data into a Lineweaver-Burk plot and calculated the *x*- and *y*-*intercept* values in secondary plots. In '$1/V_{max}^{app\text{-}g6p}$ versus $1/NADP^+$' secondary plots, the *y*-intercept (when $x = 0$) and *x*-intercept (when $y = 0$) correspond to $1/V_1$ and $-1/K_{NADP}$, respectively; and in '$(K_m/V_{max})^{app\text{-}g6p}$ versus $1/NADP^+$' secondary plots, the *y*-intercept (when $x = 0$) and *x*-intercept (when $y = 0$) correspond to K_{G6P}/V_1 and $-K_{G6P}/(K_{NADP} \cdot K_{iG6P})$, respectively. The parameters V_1, K_{G6P}, K_{NADP} and K_{iG6P} are the kinetic constants from Cleland's[24] ordered bi-bi reaction equation.

5.4. *Tb* and *Lm*G6PDH inhibition by DHEA and EA

20-fold DHEA and EA stock solutions were prepared in 50% DMSO. In the final reaction volume, the DMSO concentration was reduced to 2.5%. *Tb*G6PDH was assayed in the presence of concentrations of EA varying between 3.125 and 0.39 μM and DHEA between 20 and 2.5 μM of DHEA. *Lm*G6PDH was assayed against 50 and 100 μM of EA and DHEA, respectively. A Biomek3000 robot was used to pipette assay solutions into 96 well plates and NADPH formation rates were recorded in SpectraMax Plus^{384} (Molecular Devices). The final reaction volume was 0.2 ml per well. *Lm* and *Tb*G6PDH inhibition were assayed separately for G6P and $NADP^+$. For G6P as varying substrate, 5 μl of G6P stock solutions were loaded into wells of four plate's columns, each plate line received a different G6P concentration varying from 1250 to 9.77 μM. The reaction was initiated by addition of a mix solution containing the enzyme (2 nM), inhibitor and $NADP^+$ (0.25 mM) in *RB* reaction buffer. For $NADP^+$ as varying substrate, 5 μl of $NADP^+$ stock solutions were loaded into wells of four plate's columns, each plate line received a different $NADP^+$ solution. The final $NADP^+$ concentration varied from 250 to 1.95 μM. The reaction was initiated by addition of a mix solution containing the enzyme (2 nM), inhibitor and G6P (1.25 mM) in *RB* reaction buffer. Linear absorbance rates were recorded for 80 s. Using GraphPad Prism v.5, data were adjusted by non-linear regression to the uncompetitive inhibition equation.

5.5. *T. brucei* and *L. mexicana* viability assay

4×10^5 cells of *T. brucei* (427 strain) bloodstream forms in the initial exponential growth phase were incubated in HMI-9 medium supplemented with 10% FBS, 1% DMSO and DHEA or EA at concentrations of 4, 6, 8, 12, 16, 24, 32, 48, 64 and 96 μM for 48 h at 37 °C and under 5% CO_2. For the *L. mexicana* toxicity assay, $2x10^6$ early-metacyclic promastigotes[38] were incubated in M119 medium, supplemented with 10% FBS, 2% DMSO and DHEA or EA at concentrations of 8, 12, 16, 24, 32, 48, 64, 96, 128, 196 μM for 48 h at 25 °C. Next, 80 μl of MTS (5% PMS) from the CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) was added to 400 μl of each *T. brucei* and *L. mexicana* culture; the suspensions were distributed over a 96 well plate (100 μl per well) and incubated at 37 °C (5% CO_2) for an additional 3 h. Soluble formazan, produced by viable cells by reduction of MTS, was measured at 590 nm with a SpectraMax Plus384 spectrophotometer (Molecular Devices). Data obtained by three independent experiments were normalized and fitted to a dose–response curve using GraphPad Prism v.5 (GraphPad).

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