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Solanesyl Diphosphate Synthase, an Enzyme of the Ubiquinone Synthetic Pathway, Is Required throughout the Life Cycle of *Trypanosoma brucei*

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Ubiquinone 9 (UQ9), the expected product of the long-chain solanesyl diphosphate synthase of *Trypanosoma brucei* (TbSPPS), has a central role in reoxidation of reducing equivalents in the mitochondrion of *T. brucei*. The ablation of TbSPPS gene expression by RNA interference increased the generation of reactive oxygen species and reduced cell growth and oxygen consumption. The addition of glycerol to the culture medium exacerbated the phenotype by blocking its endogenous generation and excretion. The participation of TbSPPS in UQ synthesis was further confirmed by growth rescue using UQ with 10 isoprenyl subunits (UQ10). Furthermore, the survival of infected mice was prolonged upon the downregulation of TbSPPS and/or the addition of glycerol to drinking water. TbSPPS is inhibited by 1-[(*n*-oct-1-ylamino)ethyl] 1,1-bisphosphonic acid, and treatment with this compound was lethal for the cells. The findings that both UQ9 and ATP pools were severely depleted by the drug and that exogenous UQ10 was able to fully rescue growth of the inhibited parasites strongly suggest that TbSPPS and UQ synthesis are the main targets of the drug. These two strategies highlight the importance of TbSPPS for *T. brucei*, justifying further efforts to validate it as a new drug target.

The hemoflagellate parasite *Trypanosoma brucei* is responsible for sleeping sickness, a serious disease affecting humans and other vertebrates in sub-Saharan Africa. The main drugs used for treatment have numerous side effects, some are complicated to administer, and poor efficiency with increasing incidence of drug resistance has been reported (1). Therefore, new drugs targeting essential metabolic pathways are urgently needed.

We are interested in polyprenyl diphosphate synthases, enzymes that catalyze the elongation of isoprenoid chains through the condensation of isopentenyl pyrophosphate (a 5-carbon unit, C₅) with allylic prenyl pyrophosphates (2) to produce chains of variable length. The detection of prenylated proteins showed that short isoprenoid chains, both farnesyl and geranylgeranyl, are indeed attached to proteins in this protist (3, 4). Activities of two key enzymes of this pathway in *T. brucei*, namely, farnesyl diphosphate synthase and farnesyl transferase, have been characterized (5, 6, 7). Moreover, promising inhibitors of farnesyl diphosphate synthase with antiparasitic activities have been tested *in vitro* (8, 9, 10) and *in vivo* (11).

On the other hand, enzymes synthesizing longer isoprenoid chains have so far not been thoroughly studied in trypanosomatids (9, 12). Their product is likely to be incorporated into ubiquinone (UQ), which has a central role in respiration of *T. brucei* and has two well-studied metabolically distinct stages in its life cycle. The bloodstream form (BSF), present in vertebrate blood, respire solely via trypanosome alternative oxidase (TAO), while the procyclic form (PCF), which occurs in the tse-tse fly vector, uses both TAO and cytochrome *c*-containing respiratory chain enzymes (for reviews, see references 13 and 14). Although UQs of different lengths have been found in various parasitic protists (12, 15), so

far only UQ9 was detected in the BSF of *T. brucei* via mevalonate, its labeled precursor (16, 17).

Due to the importance of UQ in the parasite's metabolism, we decided to study TbSPPS (the *T. brucei* solanesyl diphosphate synthase), which is responsible for the synthesis of 9 isoprenyl subunit chains. Alterations in the UQ level may affect oxygen consumption, reoxidation of NADH, and the ATP pool. Indirectly, the mitochondrial membrane potential in PCF, which is produced via the respiratory chain as in most other aerobic eukaryotes, could decrease. The situation is different for the mammalian-infective BSF cells, which uniquely generate the same potential through the ATP-consuming reverse action of ATP synthase (18). Since UQ participates in the regeneration of the NADH required for ATP synthesis in the glycosomes, the shortage of reduced cofactor is likely to decrease the ATP level in this compartment, as well as in the cytoplasm and mitochondrion.

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Reactive oxygen species (ROS) are mostly generated at a low rate as a by-product of the respiratory chain, mainly from complexes I and III (19, 20). Having a central position in the respiratory chain, UQ receives in a typical cell electrons from complexes I and II and, if present, from alternative NADH dehydrogenase. While both the presence and activity in the *T. brucei* PCF of complex II and rotenone-insensitive alternative NADH dehydrogenase are undisputed (13, 14, 21, 22), both the composition (23, 24) and activity of complex I seem to be highly unusual (25, 26). Diminishing the cellular concentration of UQ could then favor an increase of the reduced NADH pool with parallel formation of ubisemiquinone, facilitating the deviation of electrons to oxygen with consequent mitochondrial ROS formation. A lower amount of UQ could also affect its function in membranes outside the mitochondrion, where it reduces lipid peroxyl radicals and radical scavengers like α -tocopheryl and, together with the cytochrome b_5 reductase, whose gene is present in the *T. brucei* genome, even assists in extracellular ascorbate stabilization (27). Hence, the depletion of the UQ pool in *T. brucei* may disrupt the redox equilibrium, increasing ROS through a multifaceted action.

Indeed, the downregulation of the mitochondrion-confined TbSPPS (28) triggered serious metabolic effects in both life stages of *T. brucei*. These effects were mimicked in the wild-type cells by the TbSPPS bisphosphonate inhibitor 1-[(*n*-oct-1-ylamino)ethyl] 1,1-bisphosphonic acid (compound 1) (9). *In vivo*, infected mice displayed longer survival when TbSPPS was ablated by RNA interference (RNAi), confirming its importance in the metabolism of the parasite.

MATERIALS AND METHODS

Materials. Compound 1 was prepared as previously described (9). Nickel-nitrilotriacetic acid-agarose was obtained from Qiagen, and paraquat, dihydroethidium, and UQ10 were provided by Sigma. UQ10/ β -cyclodextrin (a kind gift from A. Šmidovnik) is a complex of 7.5% UQ10 (Bulk Medicines & Pharma, Germany) and β -cyclodextrin (Xi'an HongChang Pharma, China). Tetramethylrhodamine ethyl ester (TMRE) was purchased from Molecular Probes, and Mitotracker Deep Red and CellTiter-Glo reagent were obtained from Invitrogen and Promega, respectively.

DNA sequencing and bioinformatics. The entire coding sequence of the TbSPPS gene was PCR amplified from genomic DNA (strain 29-13) using the primers PreBru1 (5'-CCTCGAGATCTATGCACCGTGCTAAT ATTATAT-3') and PreBru2 (5'-CCAAGCTTCACAATCCCCGTGTCA GG-3'), which introduced BglII and HindIII restriction sites, respectively, for convenient cloning into the p2T7-177 RNAi and expression vectors. In addition, primer PreBru1 contains an XhoI restriction site, which was used for cloning into the pZJM RNAi vector. All constructs were verified by sequencing. Homology searches were performed using BLAST or GeneDB, and sequences were aligned using ClustalX 1.81. The molecular weight and isoelectric point were obtained from the ExPASy server (cn.expasy.org).

Determination of EC₅₀s. Parasites were adjusted to an initial concentration of 5×10^4 BSF or 1×10^6 PCF ml⁻¹ in 200 μ l medium and loaded into sterile 96-well plates. Two-fold serial dilutions of compound 1 (boiled to ensure complete dissolution and sterility) were added to duplicate wells. After 3 days, cells in all wells were counted by using a Neubauer hemocytometer. Each assay was repeated three times. The EC₅₀ (effective concentration for half-maximal growth inhibition) was determined using the CompuSyn software (Combosyn) (29).

Plasmid constructs, transfections, cloning, RNAi induction, and cultivation. The full-size TbSPPS gene (1,080 bp) was cloned into the pZJM (30) and p2T7-177 (31) vectors by using the XhoI, BglII, and HindIII sites included in the primer sequences. *T. brucei* PCF 29-13 and BSF single marker (SM) cell lines were transfected with the linearized

constructs and selected as described elsewhere (32, 33). The PCF flagellates were cultured at 27°C in SDM79 medium supplied with 15 μ g ml⁻¹ neomycin G418 and 50 μ g ml⁻¹ hygromycin and diluted to 10^6 cells ml⁻¹ every other day, while BSF were kept at 37°C in HMI-11 medium with 2.5 μ g ml⁻¹ G418 and 5% CO₂ and diluted to 10^5 cells ml⁻¹ every other day. Phleomycin-resistant transfectants of both stages (2.5 or 1.3 μ g ml⁻¹) were cloned by limiting dilution, and RNAi was induced by adding 1 μ g ml⁻¹ tetracycline to the medium. Lister 427 PCF (29-13) and BSF (90-13) cell lines (34) were used for the inhibition experiments. Cell concentrations were determined by using a Neubauer hemocytometer or the Z2 Coulter Counter.

Northern and Western blot analyses. Total RNA was isolated using TRIzol (Sigma), and 10 μ g of RNA per lane was loaded on a 1% formaldehyde agarose gel, blotted, linked to the membrane, and hybridized with a radiolabeled probe under conditions described elsewhere (35). Total cell lysates were separated on 12% SDS-PAGE gels, transferred to membranes, and probed with polyclonal antibodies against RNA binding protein 16 (RBP16; kindly provided by L. Read) and against TbSPPS at 1:1,000 dilutions (28). Appropriate secondary antibodies (1:2,000; Sevapharma, Czech Republic) coupled to horseradish peroxidase were visualized using an enhanced chemiluminescence kit (Pierce) according to the manufacturer's protocol.

Measurement of respiration rate, $\Delta\Psi_m$, and reactive oxygen species. Oxygen consumption of both stages was measured as described elsewhere (35, 36). Changes of ROS or the mitochondrial membrane potential ($\Delta\Psi_m$) were determined using the FACSCalibur or the FACSARIA flow cytometer instruments (Becton, Dickinson) after the addition of dihydroethidium, TMRE, or Mitotracker Deep Red to the cell suspensions (10^6 cells ml⁻¹) at final concentrations of 15 μ M, 250 nM, or 500 nM, respectively, with carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) at a final concentration of 20 μ M used as a control. A total of 10,000 events were acquired in the region previously established as that corresponding to the parasites. Data were analyzed with a one-way analysis of variance (ANOVA). Significant differences among means were identified by Tukey and Dunnett posttests, and a *P* value of ≤ 0.05 was adopted as the minimum criterion of significance. Statistical analyses were performed using the GraphPad software. Alterations in the fluorescence were quantified as the percentage of its variation compared with untreated parasites used as a control. The data shown in the graphs of the figures are expressed as means $\pm$ standard deviations of at least two independent experiments.

***In vivo* infectivity and glycerol treatment.** Mice had food and fresh water available *ad libitum*. The housing conditions, care, handling, and euthanasia method were approved by our institution's Animal Ethics Committee. To determine infectivity of trypanosomes depleted for TbSPPS, four groups of CD-1 mice (5 animals each) were infected intraperitoneally with 100,000 BSF RNAi cells. In their drinking water, the first group received 1 mg ml⁻¹ doxycycline (AppiChem) sweetened with 50 mg ml⁻¹ sucrose, starting 2 days before the infection. The second group received 5% glycerol in the drinking water, while the third group received both glycerol and doxycycline. The control group was supplied with pure drinking water. The survival was recorded at least twice a day.

High-performance liquid chromatography. To calibrate the high-performance liquid chromatography (HPLC) column, the following molecules were run: UQ8 extracted with hexane from *Escherichia coli*, UQ9 isolated from *T. brucei*, and commercially available UQ10. Treated (1 μ M compound 1) and untreated BSF were pelleted and diluted in 1 ml methanol. As an internal standard, a known amount of UQ10 was added. The samples were extracted twice with 1 ml hexane. Both extraction fractions were pooled, dried under nitrogen flow, and dissolved in hexane. Samples were analyzed in an HPLC Waters apparatus with a Supelco C₁₈ column at 0.7 ml min⁻¹ flow. The mobile phase was methanol:hexane, 80:20 (vol/vol), isocratic, the loop was 5 μ l, and the detection was at 275 nm. All solvents were HPLC grade. The amount of UQ9 was quantified from the area under the curve by comparison with the UQ10 standard.

Measurement of ATP content. An equal volume of the CellTiter-Glo reagent (Promega) was added to *T. brucei*, and after a 10-min incubation, luminescence was read in a Glomax multidetection system (Promega). The signal was directly related ($r^2 = 0.99$) to the cell number per well, in the range of 30,000 to 500,000 cells. The luminescence produced by serum-supplemented HMI-11 medium alone was 2 orders of magnitude lower than that produced by the flagellates.

RESULTS

TbSPPS gene. The *Trypanosoma cruzi* TcSPPS gene, used as a query, identified a single *T. brucei* gene (Tb09.160.4300), which encodes a protein with a calculated molecular mass of 39.2 kDa and an isoelectric point of 6.12. The alignment of TbSPPS and TcSPPS revealed in both proteins the presence of seven regions related to catalysis or binding (37, 38) (see Fig. S1 in the supplemental material). The TbSPPS gene, identical in strains 29-13 and TREU927/4, is highly conserved between *T. brucei* and *T. cruzi*, as there was 66% and 68% identity (83% similarity) at the nucleotide and amino acid levels, respectively (see Fig. S1). Additionally, alanine occupies position -5 before the first and second aspartate-rich motif (see Fig. S1), allowing elongation of the isoprenoid chain longer than C₁₅ (39).

Inhibition of TbSPPS expression by RNAi. To assess the importance of the protein for the parasite's metabolism, PCF and BSF cells were transfected with the pZJM and pT7-177 RNAi vectors, respectively, each of which bore a full-length TbSPPS gene. First, total RNA was isolated from the noninduced and RNAi-induced PCF clonal cell line and analyzed by Northern blotting. In the parental 29-13 cells, TbSPPS was abundantly transcribed (band of ~1.9 kb) (Fig. 1A; see also Fig. S1 in the supplemental material), but, probably due to a small amount of leakage of the T7 promoter, less TbSPPS mRNA was present in the noninduced cells (clone 4). This effect was likely reflected also by the slight growth inhibition of the noninduced cells compared to the 29-13 parental cells (Fig. 2A). Upon induction of RNAi with tetracycline, the TbSPPS mRNA was undetectable after 2 days, with a concurrent massive appearance of double-stranded RNA (Fig. 1A), yet a slow growth phenotype of PCF started only from day 7 (Fig. 2A). Based on the growth curve, day 6 post-RNAi induction was selected for all subsequent experiments. At this time point, the levels of the TbSPPS mRNA and corresponding protein became undetectable by Northern and Western blot analyses (Fig. 1A), confirming the high efficiency of RNAi.

The viability of the BSF cells was also compromised upon RNAi induction. As revealed by Western blot analysis, TbSPPS was equally abundant in the parental SM and the noninduced cells (clone 6), while the protein was downregulated already on day 3 post-induction (Fig. 1B). As judged by the amount of the protein, there seemed to be no leakage. Although the disappearance of TbSPPS upon RNAi induction was not complete, growth inhibition started already on day 2 (Fig. 2B).

Diminished O₂ consumption. As shown in Fig. 3, respiration of the noninduced PCF cells represented about 90% of that of the parental cell line and remained the same during the first few days after RNAi induction. However, on day 6, the oxygen consumption rate of the RNAi-induced cells dropped to approximately 60%, in correlation with the appearance of the growth phenotype. The diminished O₂ consumption then lasted till day 10 postinduction, when the measurement was finished (Fig. 3). Cyanide (KCN) and salicylhydroxamic acid (SHAM), inhibitors of the cyto-

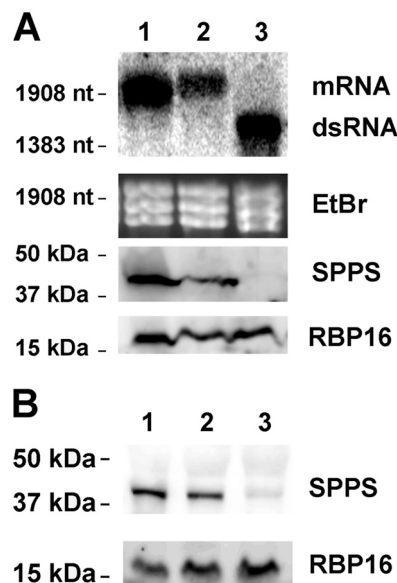


FIG 1 Northern and Western blot analyses of the TbSPPS *T. brucei* RNAi cell lines. (A) Effects of TbSPPS RNAi on mRNA and protein levels in the procyclic stage (clone 4). Total RNA and protein were extracted from parental 29-13 cells (lane 1), noninduced cells (lane 2), and procyclic cells on day 6 after RNAi induction (lane 3). The two upper panels show Northern blot analysis results with the full-length TbSPPS gene used as a probe. Ethidium bromide-stained rRNAs was used as a loading control. The two lower panels show Western blot analysis results showing expression of the TbSPPS protein in the same cell lines as in the RNA panels. The target protein was detected with specific polyclonal anti-TbSPPS antibodies. Antibody against RBP16 was used as a loading control. (B) Effects of TbSPPS RNAi on mRNA and protein levels in the bloodstream stage (clone 6) on day 3 after RNAi induction. Total protein was extracted from parental SM cells (lane 1), noninduced cells (lane 2), and bloodstream cells 3 days after RNAi induction (lane 3). TbSPPS and RBP16 were detected as described for panel A.

chrome *c* oxidase (complex IV) and TAO, respectively, were used to discriminate between the oxygen consumption of each pathway. Upon RNAi induction, no switch from one pathway to the other was observed, indicating that the decreased oxygen consumption rate was caused by both of them (Fig. 3A; also see Fig. S3A to E in the supplemental material). On the other hand, the oxygen consumption of BSF, which rely solely on TAO, dropped on day 3 post-RNAi induction to 50%, compared to the parental and noninduced parasites (Fig. 3B; see also Fig. S3F).

Inhibition of BSF RNAi cells by glycerol or compound 1. Under hypoxic or anaerobic conditions, glycerol-3-phosphate and ADP accumulate within the glycosomes, causing the glycerol kinase to operate in reverse and excrete glycerol (40). Under these conditions, exogenous glycerol added to the medium becomes a toxic metabolite, as it may diffuse into the cells, inhibiting the glycerol kinase activity and preventing NAD⁺ regeneration (41). Since the ablation of TbSPPS by RNAi will decrease the function of the glycerol-3-phosphate shuttle, the addition of glycerol should further enhance the ensuing phenotype. Indeed, while the addition of 4 mM glycerol to the HMI-11 medium within 5 days had just a mild inhibitory effect on the parental BSF cells (3.4 times slower growth) and the noninduced TbSPPS BSF cells (5.2 times slower growth), on their RNAi-induced counterparts the effect was dramatic (48.7 times slower growth) (Fig. 4A).

Next, we examined the effect of 1-[(*n*-oct-1-ylamino)ethyl]

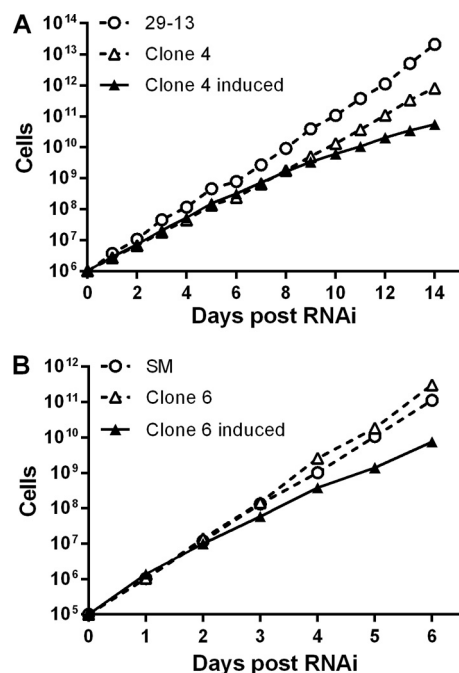


FIG 2 Effects of TbSPPS RNAi on cell growth of the procyclic form (A) and bloodstream form (B) of *T. brucei*. Cell densities (in cells ml^{-1}) of procyclic forms and bloodstream forms were measured and diluted as described in the text. The total cell numbers were calculated and plotted on a logarithmic scale on the y axis over 14 days (A) or 6 days (B). Clonal procyclic form (A) and bloodstream form (B) parasite growth in the absence or presence of 1 mg ml^{-1} tetracycline, which induces RNAi, is indicated in the graphs. The growth data for parental procyclic (strain 29-13 [A]) and bloodstream (SM [B]) forms are also shown.

1,1-bisphosphonic acid, termed here compound 1, which is a TbSPPS bisphosphonate inhibitor (Fig. 4B). After the addition of compound 1 ($1 \mu\text{M}$), BSF cell (SM) oxygen consumption dropped within 24 h to 30% of that of the nontreated parasites (Fig. 3B; also see Fig. S3F in the supplemental material), but without causing a severe growth defect (Fig. 4C). When tested on the RNAi-induced cells, strong growth inhibition occurred in the presence of the same concentration (lower than the EC_{50} [see below]). Cells grew normally for the first 2 days but died suddenly on day 3 (Fig. 4C). It should be noted that this effect could not be mimicked even by treating the parental BSF with the simultaneous addition of compound 1 and 4 mM glycerol (22.9-fold growth inhibition), suggesting that a minimal amount of the active enzyme is sufficient to support growth.

Measurement of ROS, mitochondrial membrane potential, and effects of paraquat treatment. Mitotracker and TMRE are fluorophores sensitive to the mitochondrial membrane potential ($\Delta\Psi_m$) that stain functional mitochondria. Flagellates from both stages depleted for TbSPPS did not show any significant variation of the potential compared to the parental cells.

To follow another possible outcome of the disruption of the respiratory system, we measured the generation of ROS by using dihydroethidium. Indeed, in the PCF cells, ROS increased continuously, reaching a maximum between days 6 and 8 (Fig. 5A). RNAi-induced BSF did not show any change in fluorescence upon addition of dihydroethidium, suggesting no changes in ROS generation (data not shown). The different phenotypes between the

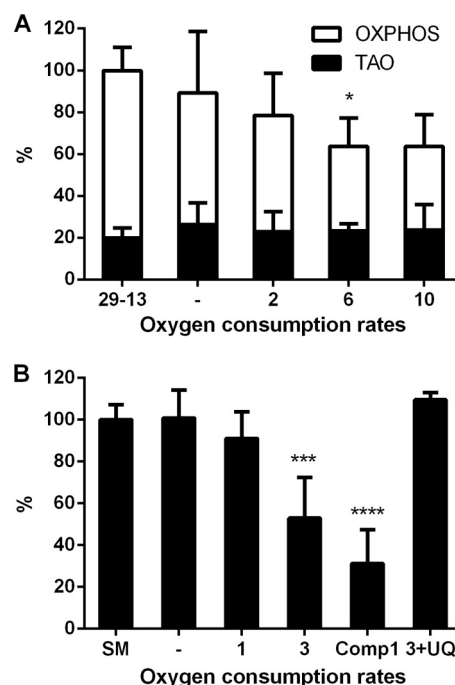


FIG 3 Effects of TbSPPS RNAi on the oxygen consumption rate in the procyclic (A) and bloodstream (B) forms of cells. (A) For procyclics, the relative contributions of the alternative pathway via TAO and of the cytochrome-mediated pathway (OXPHOS) were measured in parental 29-13 cells, noninduced cells (-), and cells at 2, 6, and 10 days after RNAi induction. The amount of O_2 consumption inhibited by KCN (0.1 mM) reflected the capacity of the cytochrome-mediated pathway, while the amount inhibited by SHAM (0.03 mM) represented the TAO activity. The noninhibited residual oxygen consumption was taken as zero. The means and standard deviation values of three experiments are shown. (B) In the absence of the cytochrome-mediated pathway in the bloodstream-form cells, all respiration is mediated by TAO. Oxygen consumption was measured in parental SM cells, noninduced cells (-), cells after 1 or 3 days RNAi induction, cells after inhibition by compound 1 ($1 \mu\text{M}$ for 24 h), and an RNAi cell group supplied with UQ10. Statistical significance levels compared to the control group are indicated by asterisks: *, $P < 0.05$; ***, $P < 0.0005$; ****, $P < 0.00005$.

two cell forms suggested that the dramatic increase of ROS in the PCF flagellates was likely generated by the disruption of the respiratory chain, which is active only in this life cycle stage.

To corroborate this result, paraquat, a reagent catalyzing ROS formation (42), was added at concentrations ranging from 0.5 to 2 μM to the noninduced and RNAi-induced PCF on day 5. Twenty-four hours later, an increment in ROS production that lasted several days was detected by flow cytometry (data not shown). The effect was paralleled by significant growth inhibition on day 8 observed in the paraquat-treated RNAi-induced cells compared to their equally treated noninduced counterparts (Fig. 5B). Thus, the ROS boost in knockdown cells was responsible for their increased sensitivity to paraquat.

Rescue of RNAi by exogenous UQ and *in vivo* infections. Addition of UQ, the downstream product of SPPS, to the medium should alleviate the effect of RNAi-mediated depletion of TbSPPS. To increase its hydrophilicity and bioavailability, UQ is usually provided bound to other compounds. We used a complex of UQ10 with β -cyclodextrin, a molecule widely used by the pharmaceutical industry for encapsulation (43). Cells ablated for TbSPPS were subjected to three different concentrations (1, 10,

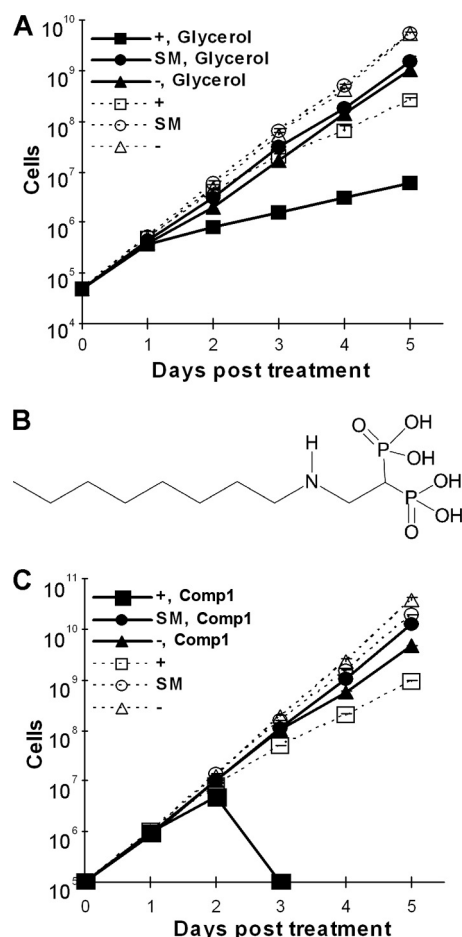


FIG 4 Growth curve of bloodstream transfected cells in the presence of glycerol or compound 1. (A) The addition of 4 mM glycerol to the medium had an inhibitory effect on TbSPPS knockdown after RNAi induction (+, glycerol), while only a mild effect was observed for the SM parental cells (SM, glycerol) and noninduced cells (-, glycerol). The same cell lines were grown in the absence of glycerol as controls. (B) Chemical structure of 1-[(n-oct-1-ylamino)ethyl] 1,1-bisphosphonic acid (compound 1), a potent inhibitor of the enzymatic activity of TbSPPS. (C) The addition of 1 μ M compound 1 to the medium was lethal for the RNAi-induced TbSPPS knockdown cells (+, Comp1), while just a very small effect was observed for the SM parental cells (SM, Comp1) and the noninduced cells (-, Comp1). The same cell lines were grown in the absence of compound 1 as controls. The experiment was repeated three times, and a representative curve is shown.

and 90 μ M) of the above-mentioned compound added to the cultivation medium. The growth of parental cells (SM) was considered 100%. In noninduced BSF cells, there was no effect of any of these concentrations on growth (89 to 95% growth). While RNAi-induced parasites (56% growth) were marginally affected upon the addition of 1 μ M UQ- β -cyclodextrin (65% growth), a significant rescue (79% and 85% growth) was observed in the presence of these compounds at 10 μ M and 90 μ M, respectively (see Fig. S2 in the supplemental material). After the addition of UQ10, the oxygen consumption returned to normal compared to the parental and noninduced parasites (Fig. 3B; also see Fig. S3F).

Next, we tested infection of animals with the transfected parasites. In humans, ingested glycerol can raise the serum concentration to 20 mM, from the normal 0.05 mM level (44). In *T. brucei*-infected animals, glycerol added to the drinking water improved

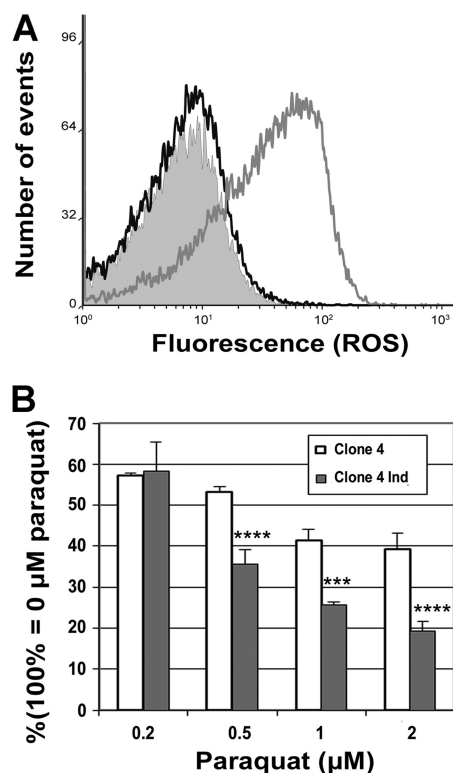


FIG 5 Generation of reactive oxygen species (A) and paraquat treatment (B) in TbSPPS procyclic cells. Experiments were performed at least twice with triplicate samples. (A) Parental 29-13 cells (area under black line), noninduced cells (gray area), and procyclics 6 days after RNAi induction (area under gray line) were incubated in the presence of 5 mg ml⁻¹ dihydroethidium for 30 min. The fluorescence distributions, measured by flow cytometry, were plotted as frequency histograms. (B) Growth of procyclic form noninduced cells (clone 4) or cells induced by RNAi for 5 days (clone 4 Ind), incubated for 3 additional days in the presence of 0.2, 0.5, 1, or 2 μ M paraquat. The growth of cells in the absence of paraquat, either noninduced or RNAi induced, was considered to be 100%. Statistical significance levels for comparisons between the two groups are indicated by asterisks: ***, $P < 0.0005$; ****, $P < 0.00005$.

the protective effect of ascofuranone (45). Four groups of mice, each composed of five animals, were infected with the same dose of 100,000 BSF RNAi transfectants and treated with different substances, with their survival rate being recorded. Two replications of this experimental setup were done with similar results, and one of them is shown (Fig. 6). While 4 days was the average survival of animals in the control group supplied with plain drinking water, the survival rate increased significantly to 7 and 7.6 days for mice drinking water containing glycerol or doxycycline, respectively. Moreover, the longest average survival of 11.2 days was recorded for animals supplied with drinking water containing both substances (Fig. 6).

Metabolic effects of compound 1 on *T. brucei*. Compound 1 was shown to be a potent inhibitor of the *T. cruzi* SPPS, with an EC₅₀ of 250 nM (9). Due to similarities in the active site and in the enzymatic mechanism of type E-polyprenyl diphosphate synthases, we hypothesized that compound 1 could also be active against the TbSPPS protein. Indeed, it affected also the growth of both *T. brucei* life forms, with EC₅₀ of 2 μ M for BSF as compared to EC₅₀ of 50 μ M for PCF.

Since TbSPPS was predicted to synthesize the isoprenoid chain

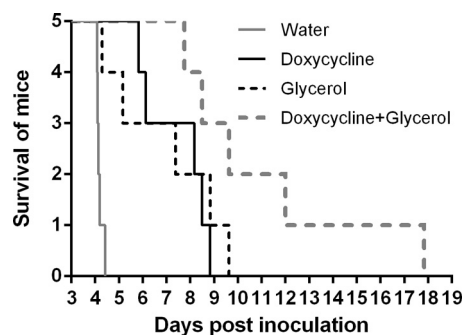


FIG 6 Survival of mice infected with TbSPPS RNAi transfectant cells was prolonged upon the addition of glycerol and doxycycline. Drinking water available to four groups of mice, each consisting of five individuals, was either pure (water) or supplemented with 1 mg ml⁻¹ doxycycline sweetened with 50 mg ml⁻¹ of sucrose, 5% glycerol, or both doxycycline and glycerol. The survival of mice was followed on a daily basis.

of UQ9, the expected effect of compound 1 treatment should be the depletion of the UQ pool. The BSF parasites were treated with the inhibitor at a concentration close to its EC₅₀, and UQ was extracted and separated by HPLC. As anticipated, a significant reduction of the UQ9 pool (90%) was observed under these conditions (Fig. 7A), as compared to extraction standard (UQ10).

UQ mediates the transfer of electrons that, through the respiratory chain and/or the glycerol 3-phosphate: dihydroxyacetone-phosphate shuttle, are passed to oxygen as the final acceptor. We have shown the oxygen consumption of cells incubated with compound 1 in Fig. 3. When increased concentrations of compound 1 were used, the respiration could even drop down to 13% in BSF and 16% in PCF, as compared to the non-treated cells. As with targeting TbSPPS by RNAi, there was no switch from KCN-sensitive to SHAM-sensitive respiration in the inhibited parasites (see Fig. S3). Trypanosomes reoxidize their NADH pool through respiration. After inhibition by compound 1, the reduced rate of reoxidation could be insufficient to provide enough ATP by glycolysis. We have measured the ATP pool in BSF using a luminescent assay. The results were expressed in luminescent arbitrary units (lau) per 5 × 10⁴ BSF cells. In comparison with untreated cells (1.4 × 10⁵ ± 28000 lau), treatment with 2 μM compound 1 lowered the signal to 6.75 × 10⁴ lau, which represents a 51% decrease.

We then tested the ΔΨ_m variation. Low concentrations of compound 1 produced a mild increase of the potential, while concentrations exceeding the respective EC₅₀ caused its slight decrease in both life stages, being significant (10–20%) only in PCF (Fig. 7B). Dissipation of 80% of the potential by the addition of the uncoupler CCCP served as a negative control. As respiratory chain is a major source of ROS (46), any of its alterations are expected to generate more ROS. When compared with the single peak of the untreated cells, PCF incubated with compound 1 formed a population with 25–35% higher amount of ROS, and an additional population with less fluorescent particles (Fig. 7C). The BSF cells, which lack a functional respiratory chain, either depleted for TbSPPS or treated with compound 1 still generate a normal amount of ROS. However, similarly to PCF, higher concentrations of the inhibitor generated a second peak with a lower ROS concentration, likely representing dead or dying cells.

Next, we tested whether UQ10 could rescue the growth inhi-

bition of the 90-13 BSF cell line caused by treatment with compound 1. The inhibitor was used at a concentration of 10 μM, which is lethal for BSF within 3 days. To eliminate the possibility of the inhibitor getting trapped by β-cyclodextrin, we added commercial UQ10 alone to the medium. In spite of the limited aqueous solubility of the bisphosphonate, the lethal phenotype was fully superseded in the presence of 20 μM UQ10, as the BSF cells grew at a normal rate (Fig. 7D).

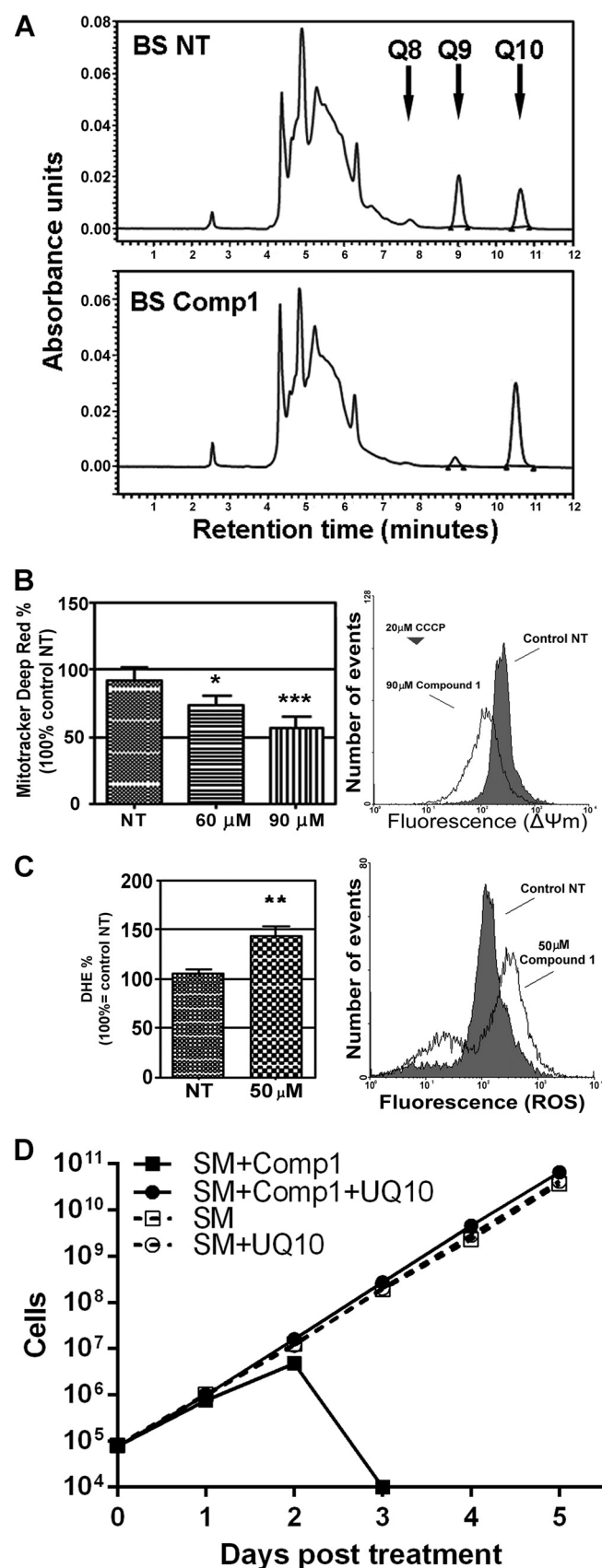
DISCUSSION

In an effort to find novel chemotherapeutic targets against pathogenic trypanosomatids, we decided to study the long chain polyprenyl diphosphate synthases. Earlier, SPPS of *T. cruzi* was characterized (12), with some bisphosphonate inhibitors tested against the recombinant enzyme (9). In order to further validate these enzymes as putative targets, we performed functional analysis of the corresponding protein in *T. brucei* using RNAi and inhibition by compound 1. This compound was shown to inhibit SPPS and the farnesyl diphosphate synthase of *T. cruzi* (9) and was thus an obvious candidate. The identification of TbSPPS was straightforward due to a high sequence similarity with its *T. cruzi* homologue. The protein was detected in the PCF and BSF cells and the downregulation by RNAi or the inhibition of TbSPPS affected the growth of both of them.

In most cells, UQ is involved in respiration, which is linked to other activities. Deficient respiration would be reflected in diminished oxygen consumption, which was indeed observed in PCF and BSF after RNAi induction or inhibition with compound 1. Insufficient mitochondrial (and glycosomal) NADH reoxidation would be reflected in lowered total ATP and altered generation of ΔΨ_m. The effect of the addition of glycerol to the RNAi-induced BSF highlighted a survival mechanism of cells experiencing an imbalance in the NADH/NAD⁺ ratio in glycosomes. In fact, the failure to efficiently reoxidate NADH through the glycerol-3-phosphate: dihydroxyacetone-phosphate shuttle bolstered the production of glycerol by the action of the glycerol-3-phosphate dehydrogenase and the glycerol kinase. Hence, hindering this outlet with exogenous glycerol seriously affected cell growth.

Nitrogen-containing bisphosphonates were earlier found to be effective *in vitro* and *in vivo* against *T. cruzi* without toxicity to the host cells (47). From the experiment with compound 1 on RNAi-induced *T. brucei*, it can be concluded that TbSPPS is inhibited in the same fashion as TcSPPS, a reflection of the high similarities of their respective active sites. In fact, compound 1 was more efficient than RNAi in abolishing the enzymatic activity of TbSPPS, leading to a lethal phenotype. Regarding the redox balance, a massive buildup of ROS was detected in PCF following RNAi induction, and a similar but lesser effect was seen after the treatment with compound 1. An incremented production of ROS by the respiratory chain could be controlled by mechanisms involving iron superoxide dismutases, which transform superoxide radicals into oxygen and hydrogen peroxide. As there are four isoforms of these enzymes distributed in glycosomes, cytosol, and mitochondria (48, 49), it would be of interest to address whether they are overexpressed in the TbSPPS knockdowns. In conclusion, both in the RNAi knockdown parasites as well as in those treated with compound 1, neither the generation and maintenance of ΔΨ_m nor ROS was the main cause of the phenotype triggered by tampering with TbSPPS.

Without active synthesis of the isoprenyl chain, the UQ pool,



estimated to be 0.1 nmol UQ in 10^9 BSF cells (17), should diminish according to its half-life which, however, remains undetermined in *T. brucei*. The UQ half-life varies in different organisms, ranging in rat tissues between 49 and 125 h (50), while in human blood it is about 34 h (51). The HPLC results for BSF after inhibition for 3 days confirmed a huge exhaustion of the pool, suggesting that the half-life of *T. brucei* UQ is comparable to that in other organisms. The HPLC experiment also provided additional data about the UQ9 content of *T. brucei* cells. The average value obtained was 1.825 ng/ 10^6 cells, equivalent to 2.3 nmol/ 10^9 cells. This represents 1.3 million molecules per cell, a value higher than the reported one (17) but still lower than that described for other cells. For example, hepatocytes, cells with a 50 times larger volume, contain 246 million molecules per cell (52).

Amino-bisphosphonates caused an ATP decrease in a tapeworm model (53), but the molecular mechanism has not been described. In *T. brucei*, ATP generation is probably not affected directly by the decrease of TbSPPS. PCF cells obtain the bulk of ATP by substrate-level phosphorylation (54, 55) or via oxidative phosphorylation (35). Through the depletion of NAD^+ , however, the interference may indirectly affect these ATP-producing processes. The same is likely to happen in BSF, which metabolize glucose in glycosomes (56) but depend on NADH reoxidation in mitochondria through the UQ-dependent glycerol-3-phosphate shuttle. Thus, the significant decrease of ATP plausibly caused the growth phenotype and, at longer times or higher concentrations of compound 1, the lethal outcome.

Another factor to be considered is the acquisition of UQ from the serum, which may replenish the dwindling intracellular pool, following the RNAi-mediated ablation of TbSPPS or its inhibition via a drug. In several organisms including humans, the UQ deficiency increases the uptake and transport of the exogenous UQ to mitochondria (57, 58). In the serum, UQ is normally transported by lipid particles, such as (very) low- as well as high-density lipoproteins, which can be taken up by specific cell receptors, some of which have already been described in *T. brucei* (59–61). It was also demonstrated that the parasite's growth is affected in lipoprotein-free serum or in the presence of antireceptor antibodies (59, 62).

Alone or in a complex with β -cyclodextrin, UQ10 was apparently taken up by trypanosomes, since the exogenous UQ rescued their growth, which was affected by either RNAi-mediated down-regulation or specific inhibition of TbSPPS. The complementation effect by exogenous UQ on inhibited parasites clearly pinpoints UQ biosynthesis rather than protein farnesylation as the main target of compound 1. While highlighting the importance of

FIG 7 Metabolic effects of the inhibition by compound 1 on wild-type parasites. (A) Measurement of the UQ pool. HPLC representative runs for untreated and treated bloodstreams are shown. The positions of the calibration standards are indicated by arrows. (B) The $\Delta\Psi_m$ displayed by procyclics treated with different concentrations of compound 1. Statistical analysis results and a representative experiment are presented. Asterisks indicate significant differences in comparison to the control group (untreated parasites). The arrowhead represents the position of the depolarized membrane control, CCCP. (C) ROS level in procyclics treated for 72 h with 50 μ M compound 1. The data are the means $\pm$ standard deviations of at least two independent experiments. (D) Rescue of parental SM bloodstream cells. The addition of 10 μ M compound 1 to the medium was lethal within 3 days (SM + Comp1). Further addition of 20 μ M UQ10 fully rescued cell growth. Nontreated (SM) and cells treated only with UQ10 (SM + UQ10) were used as controls.

UQ for the BSF cells, the rescue experiments results were also in accordance with those of an earlier report, in which synvinolin (simvastatin) reduced cell growth through the inhibition of the first enzyme of the mevalonate pathway, the 3-hydroxy-3-methylglutaryl coenzyme A reductase (62). This enzyme is responsible for the synthesis of sterols and isoprenoids. The addition of exogenous mevalonate or low-density lipoprotein particles, which transport some of the final products of the pathway, almost completely reverted the phenotype. Interestingly, the growth was reverted less efficiently by exogenous cholesterol alone, indicating that another essential product(s) present in the low-density lipoprotein particles, such as UQ, was depleted by the synvinolin inhibition (62).

The experiment in which transfected parasites were RNAi induced *in vivo* showed that interference with the synthesis of TbSPPS doubled the life span of the infected mice, confirming the *in vitro* results. Furthermore, as anticipated, the excess of glycerol further substantially prolonged survival of the infected animals. The experiments reported here show that TbSPPS is the main target of compound 1 and also that blocking the biosynthesis of UQ has important metabolic consequences for both *T. brucei* life stages.

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