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In Vitro and *In Vivo* Activity of Multi-Target Inhibitors Against *Trypanosoma brucei*

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Abstract

We tested a series of amidine and related compounds against *Trypanosoma brucei*. The most active compound was a biphenyldiamidine which had an EC₅₀ of 7.7 nM against bloodstream form parasites. There was little toxicity against two human cell lines with CC₅₀ > 100 μM. There was also good *in vivo* activity in a mouse model of infection with 100% survival at 3 mg/kg i.p. The most potent lead blocked replication of kinetoplast DNA (k-DNA), but not nuclear DNA, in the parasite. Some compounds also inhibited the enzyme farnesyl diphosphate synthase (FPPS) and some were uncouplers of oxidative phosphorylation. We developed a computational model for *T. brucei* cell growth inhibition ($R^2 = 0.76$) using DNA T_m values for inhibitor binding, combined with *T. brucei* FPPS IC₅₀ values. Overall, the results suggest that it may be possible to develop multi-target drug leads against *T. brucei* that act by inhibiting both k-DNA replication and isoprenoid biosynthesis.

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Author Contributions

G.Y., W.Z., R.D., E.O. and J.H.N. designed research; Y.W. and K.L. synthesized compounds; G.Y., S.B., and G.C. performed cell growth inhibition assays and *in vivo* experiments; W.Z. and Z.H. performed enzyme inhibition assays; W.Z. performed DSC experiments and computational modeling; G.H. and S.B. performed membrane potential measurements; G.Y., W.Z., R.D., E.O. and J.H.N. analyzed data. E.O. and W.Z. wrote the paper.

Notes

The authors declare no competing financial interest.

Supporting Information

Supporting Information Available: additional Figure illustrating uncoupling effect of **8**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

Sleeping sickness; Kinetoplast DNA; Farnesyl diphosphate synthase; Proton motive force; Computational modeling

Introduction

Diseases caused by trypanosomatid parasites afflict millions of individuals worldwide and are the causative agents of sleeping sickness in Africa, of Chagas disease in Latin America, and of the leishmaniases in India, the Middle East as well as in Latin America.¹ Many of the drugs used to treat these diseases are quite toxic (arsenicals, antimonials and benznidazole), or they are very expensive or difficult to administer in the field. Plus, many of the drugs that are in use are becoming ineffective due to resistance. Moreover, since there are no vaccines available to prevent these diseases, there is a continuing need for new drugs and new drug leads.

In this work, we focus on sleeping sickness, also known as human African trypanosomiasis (HAT). The disease is transmitted by the bite of the *tsetse* fly and is fatal if not treated, and resistance to currently used therapeutics is occurring.² There are two drugs used to treat the initial phase of the disease: suramin (**1**) and pentamidine (**2**), Figure 1, employed in the treatment of trypanosomiasis caused by *T. brucei rhodesiense* and *T. brucei gambiense*, respectively. Pentamidine therapy is rather toxic and the side effects can be fatal. For the treatment of the second or neurological phase, melarsoprol (**3**), an arsenical, is used for the treatment of both infections. However, this drug likewise causes severe side effects and is lethal in ~5-10% of patients. Both drugs are taken up into cells by the *T. brucei* adenosine and aquaglyceroporin-2 transporters and cross-resistance to both drugs is increasing.² A newer drug, eflornithine (**4**), alone or in combination with nifurtimox (**5**), has recently been introduced but is effective only for the treatment of *T. brucei gambiense* infections. Other, less toxic, inexpensive drugs active against infections caused by both species are thus required. There are promising leads that are in clinical trials, such as fexinidazole³ and oxaboroles,⁴ but it is unfortunately true that most clinical trials fail, so there is almost always a need for new concepts, and new leads.

Another class of leads are the diamidines. These compounds (such as DB75 (**6**) and its prodrug DB289 (**7**)) have been developed from the diamidine pentamidine and are thought to bind to AT-rich DNA (primarily kinetoplast DNA, k-DNA), but may also have effects as uncouplers,^{5,6} compounds that collapse the proton motive force and thus ATP synthesis. In our group, we recently found that other diamidines, such as BPH-1358 (NSC50460, **8**), had activity against two enzymes involved in isoprenoid biosynthesis, undecaprenyl diphosphate synthase (UPPS)⁷ and farnesyl diphosphate synthase (FPPS).^{8,9} There was potent (~100 nM) activity against *Staphylococcus aureus* UPPS⁷ as well as against *S. aureus*, both *in vitro* and *in vivo* in a mouse model of infection with 20/20 mice surviving when treated with **8**,⁷ while none survived without treatment.⁷ In later work,¹⁰ we found that **8** also bound to an AT-rich DNA dodecamer duplex, increasing the unfolding transition (T_m) in a DSC (differential scanning calorimetry) experiment by ~11 °C. We found that we could quite

accurately ($R^2 \sim 0.89$) model *S. aureus* cell growth inhibition by using T_m and UPPS IC_{50} results together with one mathematical descriptor,¹⁰ implying multi-target inhibition. Here, we investigated the activity of each of the compounds reported earlier as *S. aureus* cell growth inhibitors for potential activity against *T. brucei* *in vitro*, the most promising compound being tested *in vivo*. We also tested all compounds for activity against *T. brucei* farnesyl diphosphate synthase (TbFPPS), since **8** has been reported to inhibit human FPPS,⁸ as well as for activity as uncouplers, since we have found that other lipophilic bases can act as potent uncouplers.¹¹

Results and Discussion

T. brucei and human cell growth inhibition results

We first investigated the activity of the sixteen compounds whose structures are shown in Figure 2 for activity against *T. brucei* bloodstream form (BSF) parasites, as well as against two human cell lines: human embryonic kidney (HEK293T) and a human hepatocellular carcinoma (HepG2), as counter-screens for toxicity. The compounds were all from the batches whose synthesis and characterization were reported previously.¹⁰ Representative dose-response results are shown in Figure 3. All EC_{50} (*T. brucei*) and cytotoxicity (CC_{50} ; HEK293T, HepG2) values obtained are shown in Table 1. As can be seen in Table 1, there are 6 compounds (**2**, **8-11**, **13**) that have promising activity with EC_{50} values of < 100 nM against *T. brucei* cell growth. The most potent compound was **8** which had an EC_{50} of 7.7 nM, essentially the same as found with pentamidine (**2**; $EC_{50} = 5$ nM). Perhaps more interesting is the observation that the activity of **8** against both human cell lines is > 100 μ M, while that of pentamidine was < 0.4 μ M. This leads to a “selectivity index” (SI), defined as:

$$SI = CC_{50}(\text{human cell line}) / EC_{50}(T. brucei)$$

of > 13000 for **8**, versus < 80 for pentamidine **2**, suggesting that **8** might be active *in vivo*, and could have less toxicity than does pentamidine (**2**).

Mechanism of action of diamidines and related compounds

How **8** and the other compounds inhibit *T. brucei* cell growth is of interest since it might eventually lead to more potent and/or selective inhibitors, something that is needed in light of the setbacks found with **7**¹² in clinical trials. All of the compounds described here that have significant activity against *S. aureus* bind to AT-rich DNA,¹⁰ and we previously found that there was a correlation between the number of inhibitor-DNA hydrogen bonds and DNA-binding activity (as determined by T_m , the shift in the maximum of the C_p -versus- T DSC thermogram upon inhibitor binding). Plus, in this earlier work¹⁰ we found that there was a correlation between T_m and *S. aureus* cell growth inhibition. Since binding of diamidines to AT-rich DNA has been implicated in the mechanism of action of other diamidine anti-bacterials¹³, as well as in the inhibition of *T. brucei* cell growth, we sought to see whether the same mechanism was involved with the compounds described here. As can be seen in Table 1, the four compounds (**8-11**) with the largest AT-rich DNA T_m values on ligand binding ($T_m - 10^\circ\text{C}$) are all potent *T. brucei* cell growth inhibitors with EC_{50} values < 100 nM, suggesting a role of DNA binding in *T. brucei* cell growth inhibition. We thus

next sought to see if **8** (the compound with the best *T. brucei* cell growth inhibition, and selectivity index) had any effects on nuclear DNA replication, kinetoplast DNA (k-DNA) replication, or both. k-DNA, located in the *T. brucei* mitochondrion, is an unique form of DNA found only in trypanosomatid parasites and is, therefore, a good drug target.

We found, using an EdU click chemistry reaction,¹⁴⁻¹⁶ that there was a complete block of *T. brucei* k-DNA replication with **8**, as shown in Figure 4A. EdU is the thymidine-analog, 5-ethynyl-2'-deoxyuridine, which when detected using click chemistry with azide labeled Alexa•Fluor 488 leads to green fluorescence in replicating DNA/k-DNA. The bisamidine DAPI (4',6-diamidino-2-phenylindole) when bound to DNA is a highly (blue) fluorescent compound, and is used to stain both nuclear and k-DNA. As can be seen in Figure 4A, in the presence of **8**, k-DNA replication in *T. brucei* is completely blocked, just as seen with the control, ethidium bromide (bottom panel in Figure 4A). These results show that the bisamidine **8** inhibits k-DNA replication, presumably by binding to AT-rich motifs in k-DNA. The kinetoplast in *T. brucei* is, however, relatively small when compared to that found in another trypanosomatid parasite, *Leishmania donovani*, and as expected, the results of the DAPI (nuclear and k-DNA) staining and the EdU click-chemistry (replicating DNA) reactions are much more readily seen with *L. donovani*, as shown in Figure 4B where clearly, as expected, **8** blocks k-DNA replication. Nevertheless, in both *T. brucei* as well as in *L. donovani*, it appears that **8** inhibits k-DNA replication but has no obvious effect on nuclear DNA replication. These results are consistent with k-DNA replication being one target for **8** in *T. brucei* cell growth inhibition. The actual number of kinetoplasts in the visual field also decreases as a function of drug concentration and incubation time, as shown in Figure 4C for *T. brucei*, indicating kinetoplast disruption.

When we compared the T_m and *T. brucei* cell growth inhibition results (using $pEC_{50} = -\log_{10} EC_{50}$ values) there was only a poor correlation ($R^2 = 0.22$, Figure 5A). One possibility for this is that there are only relatively few compounds ($n = 16$) that are being investigated. Another possibility is that since there is considerable chemical diversity amongst the 16 structures, good correlations would not be expected because transport would be very variable. To test this possibility, we added a mathematical descriptor (one of 308 we calculated using the MOE program¹⁷), finding that the correlation between predicted and experimental *T. brucei* cell growth inhibition values increased to $R^2 = 0.66$ ($p = 0.001$), Figure 5B. The best mathematical descriptor, computed by using the MOE program¹⁷, was vsurf_EWmin1. This descriptor is the lowest hydrophilic energy (and depends on both structure connectivity and conformation), and gave $R^2 = 0.60$, $p = 0.0004$. A third possibility is that multiple-targets are involved. This possibility is the one we reported¹⁰ for *S. aureus* cell growth inhibition in which both DNA-binding (major) and UPPS inhibition (minor) were implicated, but there is no UPPS in *T. brucei*. There is, however, another prenyl synthase that is a drug target in *T. brucei*,¹⁸ farnesyl diphosphate synthase, and in earlier work we found that FPPS could be inhibited by diamidines.^{8,9}

We thus next screened all sixteen compounds against an expressed *T. brucei* FPPS. All enzyme inhibition IC_{50} values are shown in Table 1. The IC_{50} values vary considerably, from ~490 nM to ~2 mM. When the *T. brucei* cell growth inhibition (pEC_{50}) results are compared with the *T. brucei* FPPS inhibition results there is essentially no correlation

(Figure 5C), but the correlation improves to $R^2 = 0.71$ with the addition of 1 computed descriptor (PEOE_VSA_FPPOS, the fractional positive polar van der Waals surface area, computed by using the MOE program¹⁷), Figure 5D. When we use both T_m and FPPS data to predict activity:

$$pEC_{50}(\text{cell, predicted}) = a \bullet pIC_{50}(TbFPPS) + b \bullet \Delta T_m + c$$

the correlation is again poor ($R^2 = 0.28$; Figure 5E), but this improves to $R^2 = 0.76$ ($p = 0.0005$) with the addition of 1 computed descriptor, as shown in Figure 5F. These results support the idea that both k-DNA binding as well as FPPS inhibition may contribute to *T. brucei* cell growth inhibition. To put these R^2 values in perspective, in earlier work¹⁹ we compared 10 sets of enzyme inhibition/cell assay results for diverse systems (anti-bacterial, anti-protozoal, anti-cancer, anti-viral) finding that on average, the enzyme/cell pIC_{50}/pEC_{50} correlations were remarkably poor, $R^2 \sim 0.30$,¹⁹ even though in most cases highly homologous series of compounds were being investigated. The correlation greatly improved by adding mathematical descriptors (such as clogP) and, on average, the R^2 values increased to $R^2 = 0.70$ (with 2 added descriptors, and large data sets). The same general approach can be used with multiple experimental descriptors, and with *S. aureus* we found an $R^2 = 0.75$ using T_m and UPPS inhibition data, which improved to $R^2 = 0.89$ with 1 computed descriptor.¹⁰ It is possible that there are alternate targets whose inhibition is being reflected in the computational descriptors, although the use of the experimental T_m and TbFPPS pIC_{50} values together with just 1 computed descriptor does give a very good prediction of activity ($R^2 = 0.76$, $p = 0.0005$). Interestingly, we also found that the IC_{50} of DB75 (**6**) against both human and *T. brucei* FPPS was $\sim 50 \mu\text{M}$. This seems a rather large value, but FPPS could be a target for this compound in *T. brucei* since it is likely to be taken up by transporters and could accumulate within the cells, contributing to the toxicity seen with the pro-drug DB289.²⁰

In addition to k-DNA and FPPS as potential targets, there are of course other possibilities, suggested by the literature. Specifically, it has been found that pentamidine (**2**) is an uncoupler of oxidative phosphorylation in isolated rat liver mitochondria,²¹ and that the mitochondrion in *T. brucei* is the target of the trypanocidal action of the diamidine DB75.²² We thus next tested the most promising lead **8** for its ability to collapse the proton motive force (here, primarily the mitochondrial membrane potential, ψ_m) in digitonin-permeabilized bloodstream form *T. brucei* (BSF) trypanosomes (Figure 6A) and procyclic form (PCF) trypanosomes (Figures 6 B-D) using the safranin method.^{23,24} We chose compound **8** since it had no effect on the growth of either of the two human cell lines ($CC_{50} > 100 \mu\text{M}$), in addition to being the most potent *T. brucei* cell growth inhibitor and as we show below, it is active *in vivo*.

Figure 6A shows that addition of $10 \mu\text{M}$ **8** decreased ψ_m , which was further reduced by addition of $8 \mu\text{M}$ FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, $2 \mu\text{g/mL}$), a potent protonophore uncoupler. Similar results were obtained with PCF, Figure 6B. *T. brucei* PCF mitochondria were able to phosphorylate ADP, as demonstrated by the small decrease in ψ_m after its addition, Figure 6C. This activity was inhibited by the ATP

synthase inhibitor oligomycin. In addition, the mitochondria were able to transport Ca^{2+} , as shown by the decrease in the ψ_m after addition of CaCl_2 , and the ψ_m returned to basal levels after addition of the Ca^{2+} -chelator EGTA, Figure 6C. Further addition of **8** followed by FCCP again collapsed ψ_m , Figure 6C. **8** collapsed ψ_m in a dose-dependent manner (Figure 6D) and **8** alone had no effect (Figure 6B). Figure S1A and S1B show the results of 3 independent experiments. These results show that mitochondria in permeabilized *T. brucei* are able to develop a ψ_m , phosphorylate ATP and transport Ca^{2+} and that **8** collapses ψ_m . These effects on the proton motive force are rapid and are very similar to those observed for SQ109 in bacterial systems¹¹ and are likely to make a contribution to **8** inhibiting cell growth.

These observations then raise the question: do some of the other compounds affect the PMF? We thus next measured the collapse in ψ_m for all compounds, but this time at just a single (5 μM) inhibitor concentration. Results are shown in Figure 7. As a control, we used FCCP. The three most potent uncouplers (from Figure 2) were all biphenyls: **13**, **14** and **8**, Figure 7, with the 6-membered amidine **13** being the most effective species, albeit less so than was FCCP, 140 versus 230 (arbitrary) fluorescence units. The 6-membered ring species **13** was more effective than was **8**, which has 5-membered amidine rings. The next most active species were pentamidine and **9**. However, we did not obtain an improved growth inhibition models using the uncoupling results, although they may be important for individual compounds.

In addition to the effects on ψ_m , we find that there are deranged mitochondrial morphologies exhibited by *T. brucei* BSF on treatment with **8**. Figure 8 shows DMSO control cells and cells treated with 500 nM **8** for 24 hrs. DAPI (blue fluorescence) stains the nuclear and k-DNA while MitoTracker® Red (red fluorescence) is used to visualize the mitochondria, and its uptake depends on ψ_m . As can be seen in Figure 8, k-DNA is localized to the mitochondrion, however, the mitochondrial morphology is disrupted by **8**, changing from a normal tubular shaped organelle to a more condensed form, particularly towards the location near the k-DNA.

These uncoupling effects are clearly interesting, but they could lead to toxicity-as might human FPPS inhibition. However, in other work¹¹ we reported that the tuberculosis drug lead SQ109 acted at least in part as an uncoupler, and this work has now been extended²⁵ to numerous other TB drug leads including adamantyl ureas, indolecarboxamides, tetrahydropyrazolopyrimidines, and the 1,5-diarylpyrrole BM212,²⁵ all of which are now thought to function primarily as uncouplers. Moreover, drugs such as niclosamide function in the same way²⁶ and are of interest as diabetes drug leads.^{27,28} We thus next sought to see if **8** had any efficacy *in vivo*, and/or if it was highly toxic.

***In vivo* activity in a mouse model of infection**

Compound **8** has the best computed SI in both cell lines and also has essentially the same activity as pentamidine (**2**), so was chosen for further investigation. We used the *T. brucei* Lister 427 mouse model of infection in which all mice die ~6 days after infection, in the absence of any treatment. In an initial set of experiments, mice were treated with **8** at 10

or 3 mg/kg i.p. for 4 days. Figure 9A shows survival as a function of time, again with PBS, pentamidine and compound **8** treated mice. All **8** treated mice survive in this infection model at both 3 and 10 mg/kg after the 4-day treatment (and over the 25 day observational period), and there was no parasitemia observed in the **2** (pentamidine) or **8** treated mice, Figure 9B. We then carried out a second series of experiments, this time at 1 and 0.3 mg/kg i.p. for 4 days. Survival and parasitemia results are shown in Figures 9C, D and indicate (when compared with the results in Figures 9A, B) that the lowest effective dose is ~3 mg/kg. There is no weight loss during mice treatment.

Conclusions

The results presented here suggest that it may be possible to develop new multi-target inhibitors of *T. brucei* cell growth that target both parasite-specific kinetoplast DNA (k-DNA) as well as isoprenoid biosynthesis (FPPS) with such “multi-target” inhibition being of general interest since it could lead to more effective and more “resistance-resistant” drugs.²⁹ Of particular interest is the observation that the lead **8** inhibits k-DNA replication, but not nuclear DNA replication. Some of the diamidine analogs also inhibited the isoprenoid biosynthesis enzyme, farnesyl diphosphate synthase. This is of interest since FPPS is an essential protein for *T. brucei* cell growth.¹⁸ Plus, other compounds acted as uncouplers, of interest since other new drug leads against bacteria have been reported to act in this way. In a mouse model of infection, all mice survived upon treatment with **8** and a similar result (with **8**) has been reported with *S. aureus* infection in which both DNA and another isoprenoid biosynthesis enzyme, UPPS, were targeted.^{7,10} **8** also acts as an uncoupler in both bloodstream and procyclic form parasites, rapidly collapsing the mitochondrial membrane potential, ψ_m . However, while **8** was a potent inhibitor of *T. brucei* cell growth, it was a poor inhibitor of the growth of two human cell lines and did not appear toxic in mice. The basic observation that k-DNA, FPPS as well as the proton motive force can be targeted is thus of general interest in the context of developing new anti-parasitic drug leads for treating infections caused by trypanosomatid parasites.

Experimental section

Ethics Statement

All animal care and therapy studies were carried out in strict accordance with the guidelines and principles established by the Korean Animal Protection Law (<http://animalrightskorea.org>). Animal use protocol # IPK-13009-1 was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Institut Pasteur Korea.

Inhibitors

Inhibitors were from the batches whose synthesis (or availability) as well as characterization were described previously.¹⁰ All compounds were 95% pure as determined by elemental analysis or analytical HPLC/MS analysis and were also characterized by ¹H NMR and HRMS.

Parasites and Cell Culture

Trypanosoma brucei brucei Lister 427 (bloodstream form) was cultivated at 37 °C with a 5% CO₂ atmosphere in HMI-9 medium supplemented with 10% fetal bovine serum (FBS). *T. brucei* was subcultured every 3 or 4 d and maintained until the twentieth passage. The HEK239T and HepG2 cell lines used in the cytotoxicity testing were cultivated at 37 °C in a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

T. brucei Growth Inhibition

T. brucei cell growth inhibition was assayed by measuring the conversion of resazurin to resorufin. Assays were performed in duplicate in 384 well plates which were seeded with *T. brucei* (2.5×10^3 cells per well). After seeding the parasites, they were exposed to the compounds for 3 d. Resazurin sodium salt (120 µM; R7017; Sigma-Aldrich, St. Louis, MO, USA) was then added and plates incubated for 5h. After incubation, the parasites were fixed with 4% paraformaldehyde and the plates analyzed in a Victor 3TM plate reader (PerkinElmer, Inc., Waltham, MA, USA) at 590 nm (emission) and 530 nm (excitation). Pentamidine was used as a reference drug in the *T. brucei* inhibition assay. The EC₁₀₀ of pentamidine was taken as the concentration that produced 100% growth inhibition.

Cytotoxicity Assay

A resazurin cytotoxicity assay was performed in duplicate with HEK239T and HepG2 cell lines. Cells (4.0×10^3 cells per well) were seeded in 384 well plates and incubated for 72 h with selected compounds. Cells were then exposed to 40 µM resazurin for 5 h to allow for conversion to resorufin by aerobic respiration. After incubation, cells were fixed with 4% paraformaldehyde and the plates read with a Victor 3TM plate reader (PerkinElmer, Inc., Waltham, MA, USA) at 530 nm (excitation) and 590 nm (emission). Chlorpromazine was used as a reference drug in the cytotoxicity assay. The EC₁₀₀ of chlorpromazine was taken as the concentration that produced 100% growth inhibition.

Protein Expression and Purification

TbFPFS was cloned and expressed as reported previously.³⁰ Briefly, DNA coding for *TbFPFS* was cloned into the pET-28a vector (Novagen, Madison, WI, USA). The recombinant plasmid was transformed into an *Escherichia coli* BL21 (DE3) host to be expressed. Bacterial clones were grown in LB medium to an O.D. 600 of 0.8, and were induced with 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) at 37 °C. After induction for 5 h, cells were re-suspended in binding buffer (500 mM NaCl, 20 mM Na₂HPO₄, pH 7.4) and incubated with 10 mg/mL lysozyme, 10 µg/mL protease inhibitor, and 1 µL/mL benzonase nuclease (Novagen) for 15 min, on ice. The supernatant was obtained by centrifugation at 15000 *g* for 1 h, at 4 °C. Lysates were applied to a nickel-chelated agarose affinity column and washed with binding buffer. Protein was eluted from the column using binding buffer containing 500 mM imidazole. The eluted fraction was desalted with a PD-10 desalting column (GE Life Sciences, Pittsburgh, PA, USA) and stored in 10 mM HEPES buffer (pH 7.4) containing 10 mM 2-mercaptoethanol.

***In vitro* Enzyme Assay**

*Tb*FPPS inhibition assays were carried out as described previously.³⁰ Briefly, the condensation of geranyl diphosphate (GPP) with isopentenyl diphosphate (IPP) catalyzed by FPPS was monitored by using a coupled colorimetric assay³¹ in 96-well plates with 200- μ L reaction mixtures containing 400 μ M methylthioadenosine (MESG), 100 μ M IPP and 100 μ M GPP in 25 mM Tris-HCl (pH 7.4), 1 mM MgCl₂ and 0.01% Triton X-100. The highest concentration of the inhibitors in the assay was 316 μ M.

Visualization of DNA Replication

After the treatment with inhibitors for 72 h, *T. brucei* BSF trypanosomes were incubated for 16 h with the thymidine analogue EdU (5-ethynyl-2'-deoxyuridine). EdU-labeled parasites were then washed with 1 $\times$ PBS and fixed in 100% cold ethanol, and completely dried. Fixed parasites were washed with 1 $\times$ PBS and incubated with Alexa Fluor® 488 Azide (Invitrogen, Grand Island, NY, USA) under Cu(I)-catalyzed click reaction conditions (100 μ M of ascorbic acid and 1 μ M of CuSO₄). EdU-labeled parasites were counterstained with 10 μ g/ml DAPI before mounting in VECTASHIELD® mounting medium (Vector Laboratories, Burlingame, CA, USA). The images were analyzed using a Nikon Eclipse 90i fluorescence microscope (Nikon, Tokyo, Japan) and captured with a digital camera (DS-1QM, Nikon).

Visualization of Mitochondrial Membrane Potential

After the compound treatment for 24 h and 48 h, then parasites were incubated with 200 nM of Mitotracker® Red CMXRos (Invitrogen) for additional 30 min. BSF trypanosomes were washed in cold PBS and fixed with 3% PFA in PBS at 4 °C for 1 h. Fixed parasites were washed with PBS, and stained with 10 μ g/ml of DAPI before mounting in VECTASHIELD® mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were analyzed with a Nikon Eclipse 90i fluorescence microscope (Nikon, Tokyo, Japan) and captured with a digital camera (DS-1QM, Nikon).

***In vivo* Experiments**

BALB/C mice were infected with *T. brucei brucei* Lister 427 (3×10^4 cells) by i.p. injection. Mice were divided into groups (n = 5), and drug treatment carried out for 4 consecutive days by administering 10, 3, 1 or 0.3 mg/kg of compound **8** i.p. Parasitemia and survival were evaluated daily for 25 days. Mice showing impaired health status and/or with a parasite load > 10^8 cells per ml of blood were euthanized.

Analysis of Mitochondrial Membrane Potential

The mitochondrial membrane potential *in situ* was analyzed spectrofluorometrically by using safranin as the probe.^{23,24} *T. brucei* PCF and BSF trypanosomes were incubated at 28 °C in reaction buffer (125 mM sucrose, 65 mM KCl, 10 mM HEPES–KOH buffer, pH 7.2, 1 mM MgCl₂, 2.5 mM potassium phosphate) with additions as described in the Figure legends. Fluorescence changes were monitored on a Hitachi 4500 spectrofluorometer (excitation wavelength = 496 nm; emission wavelength = 586 nm).

Computational Aspects

All mathematical modeling was performed in R (<http://www.R-project.org>). Descriptors were calculated by using MOE.¹⁷

Statistical Analyses

All IC₅₀/EC₅₀ values were measured in duplicate. Dose-response curves were fitted by using a sigmoidal dose-response equation with a variable slope using GraphPad Prism 6 Software (GraphPad Software, San Diego, CA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FPPS	farnesyl diphosphate synthase
UPPS	undecaprenyl diphosphate synthase
BSF	bloodstream form
PCF	procyclic form
HEK	human embryonic kidney
human Hep	hepatocellular carcinoma
Tb	<i>Trypanosoma brucei</i>
SI	selectivity index
IC₅₀	half maximal inhibitory concentration
EC₅₀	half maximal effective concentration
CC₅₀	half maximal cytotoxicity concentration
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
MESG	7-methyl-6-thioguanosine
GPP	geranyl diphosphate
IPP	isopentenyl diphosphate
FPP	farnesyl diphosphate

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Figure 1.
Structures of some *T. brucei* cell growth inhibitors.

Figure 2.
Structures of 16 compounds investigated here as *T. brucei* cell growth inhibitors.

Figure 3.

Representative dose-response results for some compounds of interest in *T. brucei*, HEK293T and HepG2 (human cell) growth inhibition, together with computed selectivity index (SI) values ($SI = CC_{50} \text{ (human cell line)} / EC_{50} \text{ } T. brucei$). Experiments were performed in duplicate.

Figure 4.

Effects of **8** on nuclear and k-DNA replication for *T. brucei* (A, C) and *L. donovani* (B). Shown at the top in A, B are control (DMSO vehicle) results with DAPI and EdU. DAPI stains all DNA and the bound compound exhibits a blue fluorescence. EdU is used to detect replicating DNA and fluoresces green. In the DMSO controls, both nuclear (n) and k-DNA (k) replicate in both organisms (green). In the middle row panels in A, B, cells are treated with **8**, which blocks k-DNA replication, so the (replicating) kinetoplast k-DNA signal is not observed (blue). The same k-DNA replication-inhibition result is found with the DNA intercalator ethidium bromide, shown in the bottom panels of the Figure. Scale bar, 2 μ m. (C) Shows the number of kinetoplasts in the visual field as a function of **8** concentration (from 39 nM to 626 nM), and incubation time (48h or 72h). Values are means $\pm$ SD of duplicate experiments.

Figure 5.

Plots between experimental *T. brucei* cell growth inhibition (pIC₅₀ values) and experimental properties, and computed growth inhibition models. (A) T_m , $R^2=0.22$, $p=0.07$. (B)

$T_m+vsurf_EWmin1$, $R^2=0.66$, $p=0.001$. (C) FPPS pIC₅₀, $R^2=0.03$, $p=0.53$. (D) FPPS pIC₅₀+PEOE_VSA_FPPOS, $R^2=0.71$, $p=0.0003$. (E) FPPS pIC₅₀+ T_m , $R^2=0.28$, $p=0.12$. (F) FPPS pIC₅₀+ T_m + PEOE_VSA_FPPOS, $R^2=0.76$, $p=0.0005$.

Figure 6.

Effects of **8** on the mitochondrial membrane potential ψ_m in digitonin-permeabilized *T. brucei*. (A) BSF trypanosomes (2×10^8 cells) were added to buffer (2 mL) containing 20 μ M EGTA, 1 mM ATP, 500 μ M sodium orthovanadate and 5 μ M safranin, and the reaction initiated with 40 μ M digitonin. **8** and FCCP were added where indicated. Grey line is baseline. (B) PCF trypanosomes (5×10^7 cells) were added to buffer (2.4 mL) containing 2 mM succinate and 5 μ M safranin, and the reaction initiated with or without (grey trace) 50 μ M digitonin. **8** (5 μ M) and FCCP (8 μ M) were added where indicated. (C) As (B) but ADP (10 μ M), oligomycin (Oligo, 2 μ g/ml), CaCl_2 (12 μ M), EGTA (200 μ M), **8** (10 μ M), and FCCP (8 μ M) were added where indicated. (D) As (B) but CaCl_2 (12 μ M), EGTA (200 μ M), various concentrations (0–30 μ M) of **8** and FCCP (8 μ M) were added where indicated.

Figure 7.

Effects of compounds on the mitochondrial membrane potential (ψ_m) in digitonin permeabilized *T. brucei* PCF trypanosomes. PCF trypanosomes (5×10^7 cells) were added to reaction buffer (1.95 mL) with additions as described in Figure 6 (B). The chart shows changes in safranin fluorescence (AU = arbitrary units) after addition of each compound (5 μ M) or FCCP (5 μ M). The results are means $\pm$ SD of three independent experiments.

Figure 8. Effects of **8** (156 and 625 nM, 24 hrs and 48hrs) on *T. brucei* cells. DAPI fluoresces blue and stains DNA; Mitotracker Red fluoresces red and is used to locate the mitochondrion (Scale bar, 5 μ m).

Figure 9.

In vivo results for *T. brucei brucei* Lister 427 mouse model of infection. (A), Mouse survival after treatment at 3 and 10 mg/kg i.p. for 4 days. (B), Parasitemia as a function of time after treatment. (C-D) Same as (A-B) but at 0.3 and 1 mg/kg i.p. for 4 days.

Table 1

Enzyme inhibition, cell growth inhibition, DSC and fluorescence results.

ID	^a <i>T. brucei</i> EC ₅₀ (μM)	^b HEK293T CC ₅₀ (μM)	^c HepG2 CC ₅₀ (μM)	^d SI HEK/Tb	^e Hep/TbSI	^f T _m (°C)	^g Fluor. A.U.	^h TbFPPS IC ₅₀ (μM)
9	0.084 (0.069-0.1)	> 200	> 200	> 2400	> 2400	24	65 ± 5.0	3.5 ± 0.072
10	0.051 (0.041-0.063)	> 100	41 (34-50)	> 2000	810	20	12 ± 2.6	1.3 ± 0.017
11	0.020 (0.015-0.026)	> 100	> 100	> 5000	> 5000	16	25 ± 3.1	9.9 ± 0.50
12	2.1 (1.8-2.4)	> 200	> 200	> 96	> 96	16	13 ± 5.8	950 ± 230
8	0.0077 (0.0063-0.012)	> 100	> 100	> 13000	> 13000	11	81 ± 2.9	27 ± 0.25
13	0.095 (0.076-0.12)	14 (10-18)	59 (45-77)	140	620	10	140 ± 16	310 ± 93
14	0.85 (0.77-0.93)	27 (21-35)	>100	32	118	7.1	110 ± 16	0.49 ± 0.0088
15	> 50	> 100	> 100	~ 2	~ 2	5.8	19 ± 4.5	21 ± 0.61
16	1.9 (1.4-2.6)	4.7 (4-5.5)	6.4 (5.8-7.2)	2.5	3.4	5.2	55 ± 2.5	18 ± 7.0
17	0.85 (0.62-1.2)	> 20	> 20	> 24	> 24	3.9	37 ± 4.4	240 ± 36
2	0.0055 (0.0052-0.0057)	< 0.4	< 0.4	< 80	< 80	2.7	64 ± 11	370 ± 7.2
18	0.42 (0.16-1.0)	14 (13-16)	33 (15-73)	35	80	0.7	18 ± 7.7	1800 ± 230
19	0.67 (0.55-0.81)	2 (1.6-2.5)	12 (9.5-15)	3	18	0.6	30 ± 3.3	45 ± 11
20	0.7 (0.66-0.74)	13 (12-15)	6.1 (5-8.7)	19	8.7	0.1	19 ± 8.3	4.4 ± 0.49
21	> 50	> 100	> 100	~ 2	~ 2	-1.0	7.1 ± 2.4	6.6 ± 2.8
22	23 (5.3-97)	> 100	> 100	> 4.4	> 4.4	-1.2	12 ± 2.6	1.9 ± 0.47

^a*T. brucei* EC₅₀ were determined from duplicate measurements by nonlinear regression analysis using GraphPad Prism 6. Values in parentheses are 95% confidence intervals.

^bHEK293T CC₅₀ were determined from duplicate measurements. Values in parentheses are 95% confidence intervals.

^cHepG2 CC₅₀ were determined from duplicate measurements. Values in parentheses are 95% confidence intervals.

^dSelective index: CC₅₀ (HEK293T)/EC₅₀ (*T. brucei*).

^eSelective index: CC₅₀ (HepG3)/EC₅₀ (*T. brucei*).

^fData from ref. 10.

^gFluorescence intensity, arbitrary units. Larger values mean stronger uncoupling effects. Results shown are the average of triplicate experiments ± standard deviation.

^h*T. brucei* FPPS IC₅₀. Data points are the average of duplicate experiment, ± standard deviation.