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Trypanocidal action of bisphosphonium salts through a mitochondrial target in bloodstream form *Trypanosoma brucei*

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ABSTRACT

Lipophilic bisphosphonium salts are among the most promising antiprotozoal leads currently under investigation. As part of their preclinical evaluation we here report on their mode of action against African trypanosomes, the etiological agents of sleeping sickness. The bisphosphonium compounds CD38 and AHI-9 exhibited rapid inhibition of *Trypanosoma brucei* growth, apparently the result of cell cycle arrest that blocked the replication of mitochondrial DNA, contained in the kinetoplast, thereby preventing the initiation of S-phase. Incubation with either compound led to a rapid reduction in mitochondrial membrane potential, and ATP levels decreased by approximately 50% within 1 h. Between 4 and 8 h, cellular calcium levels increased, consistent with release from the depolarized mitochondria. Within the mitochondria, the Succinate Dehydrogenase complex (SDH) was investigated as a target for bisphosphonium salts, but while its subunit 1 (SDH1) was present at low levels in the bloodstream form trypanosomes, the assembled complex was hardly detectable. RNAi knockdown of the SDH1 subunit produced no growth phenotype, either in bloodstream or in the procyclic (insect) forms and we conclude that in trypanosomes SDH is not the target for bisphosphonium salts. Instead, the compounds inhibited ATP production in intact mitochondria, as well as the purified F_1 ATPase, to a level that was similar to 1 mM azide. Co-incubation with azide and bisphosphonium compounds did not inhibit ATPase activity more than either product alone. The results show that, in *T. brucei*, bisphosphonium compounds do not principally act on succinate dehydrogenase but on the mitochondrial F_oF_1 ATPase.

1. Introduction

African trypanosomes are responsible for a spectrum of important human and veterinary diseases south of the Sahara, transmitted by various tsetse fly species. *Trypanosoma brucei gambiense* causes a chronic but fatal human African trypanosomiasis (HAT, or sleeping sickness) in Western and Central Africa, whereas *T. b. rhodesiense* causes a much more acute illness in Eastern and Southern Africa (Brun et al., 2010); both forms of the disease are considered almost invariably fatal if left untreated. Whereas transmission of *T. b. gambiense* appears to be almost exclusively anthroponotic, *T. b. rhodesiense* is a zoonotic parasite, with many wild and domestic animals, particularly cattle, acting as reservoirs (Welburn et al., 2001). In addition, *Trypanosoma congolense*, *T. b. brucei*, *Trypanosoma evansi* and *Trypanosoma vivax* cause animal African trypanosomiasis (AAT), inflicting a terrible burden on agriculture in the tsetse belt and, for the latter two species, also in areas far beyond the testse habitat including the Indian subcontinent and South America (Desquesnes et al., 2013; Osório et al., 2008; Swallow, 1999).

While vector control in conjunction with extensive surveillance can have highly significant local impact on the disease burden, there is no realistic prospect of a vaccine (La Greca and Magez,

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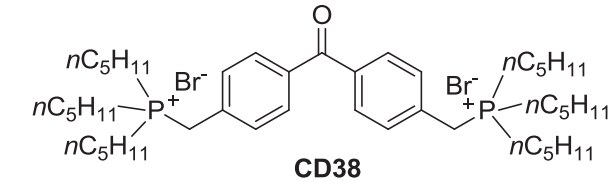
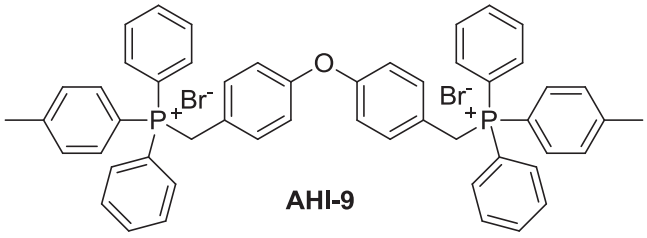
2011) and chemotherapy is practically the only option in most areas (Delespaux and de Koning, 2007; Jannin and Cattand, 2004). However, few drugs currently exist for either HAT or AAT, and these are old, suffer from severe side-effects and/or resistance, and are usually only effective against certain (sub) species or stages of the disease (Brun et al., 2010; Delespaux and de Koning, 2007). New therapeutic agents are therefore urgently necessary, preferably active against all African trypanosome species, and against both stages of the human disease. One promising lead is the class of benzyltriphenylphosphonium compounds that displays highly potent activity against rodent models of *T. b. rhodesiense* (Kinnamon et al., 1979), *Trypanosoma cruzi* (Kinnamon et al., 1977) and *Leishmania donovani* (Hanson et al., 1977) infections. Moreover, a large series of bisphosphonium salts were shown to possess strong anti-leishmanial (Luque-Ortega et al., 2010) and trypanocidal (Dardonville et al., 2015; Taladriz et al., 2012) activity in vitro. Many of these compounds showed high selectivity against the kinetoplastid parasites relative to human cell lines and, importantly, displayed no cross-resistance with existing trypanocides such as diamidines and melaminophenyl arsenicals (Taladriz et al., 2012).

The triphenylphosphonium (TPP) moiety has been used extensively as a vehicle to deliver drugs to mitochondrial targets (Cairns et al., 2015; Cortes et al., 2015; Smith et al., 2011). Among many applications, TPP has been used to deliver functional probes (Cairns et al., 2015), antioxidants (Kelso et al., 2001; Teixeira et al., 2012), anti-cancer drugs (Jara et al., 2014), and even liposomes (Benein et al., 2015) to mitochondria. TPP-linked natural compounds botulin and betulinic acid showed in vitro and in vivo activity against *Schistosoma mansoni* (Spivak et al., 2014). Crucially, TPP-mediated mitochondrial targeting appears to be generally safe, as a double-blind, placebo controlled study with human volunteers found no evidence of side-effects of the TPP-coupled antioxidant MitoQ over a 12-month period (Snow et al., 2010).

The mitochondrial accumulation of TPP-coupled drugs is driven by the strong inside-negative potential across the inner membrane of the mitochondrion. TPP, being a lipophilic cation with a highly dispersed charge is believed to diffuse freely across the inner membrane and be highly concentrated in the mitochondrial matrix, to an extent determined by the mitochondrial membrane potential Ψm and the plasma membrane potential V_m, as described by the Nernst equation (Cairns et al., 2015). Consistent with the extensive TPP literature, Luque-Ortega et al. showed that the anti-leishmanial TPP analogues targeted the parasite's mitochondrion, and proposed their principal action to be the inhibition of the succinate dehydrogenase complex (complex II) (Luque-Ortega et al., 2010). However, similar compounds also had strong activity against bloodstream form (BF) *T. brucei* (Dardonville et al., 2015; Taladriz et al., 2012), which has a much less elaborate mitochondrial metabolism, lacking for instance much of the Krebs cycle, and the cytochrome-dependent respiratory chain (Tielens and van Hellemond, 2009). Transfer of electrons to oxygen is instead mediated by a plant-like alternative oxidase (Chaudhuri et al., 1998) and the mitochondrial membrane potential is maintained by the F_oF_1 ATPase pumping protons from the mitochondrial matrix (Nolan and Voorheis, 1992; Schnaufer et al., 2005). We thus investigated whether the succinate dehydrogenase complex is expressed in BF *T. brucei*, and whether this might be the primary target for the trypanocidal activity of triphenylphosphonium salts and their analogues. We selected two compounds with strong trypanocidal activity (Taladriz et al., 2012) to represent the main two classes of aliphatic and aromatic bisphosphonium salts (Fig. 1).



CD38



AHI-9

Fig. 1. Benzophenone-derived bisphosphonium salts used in this study.

2. Materials and methods

2.1. Effect of bisphosphonium compounds on growth of BF T. brucei

The effects of AHI-9 and CD38 on growth of BF *Trypanosoma brucei brucei* strain Lister 427 were investigated by incubating cultures under standard conditions (HMI-9/10% FBS; 37 °C, 5% CO_2) in the presence or absence of 0.1 μM, 0.3 μM or 1 μM test compound for up to 72 h. Samples were taken in triplicate at the following times (h) after initiation of the experiment: 0, 4, 8, 12, 24, 28, 32, 36, 48, 52, 56, 60, 72. Cell density in the samples was determined using a haemocytometer. Compounds CD38 and AHI-9 were synthesized as reported previously (Luque-Ortega et al., 2010; Taladriz et al., 2012).

2.2. Determination of trypanocidal action using the Alamar blue assay

Fifty percent effective concentrations (EC_{50}) were determined using the fluorescence viability indicator dye Alamar Blue (resazurin sodium salt, Sigma), exactly as described (Gould et al., 2013, 2008). Serial doubling dilutions of test compounds were prepared over two rows of a 96-well plate, leaving the last well without added drug as a control, before the addition of an equal volume of cell suspension to each well. Final cell density in each well was 10^5 BF *T. brucei* per mL. The plates were incubated for 48 h (37 °C, 5% CO_2) before the addition of 20 μL resazurin solution (125 μg/mL in PBS pH7.4) per well and incubation for a further 24 h. Fluorescence was determined using a FLUOstar Optima (BMG Labtech, Durham, NC, USA) with excitation wavelength set at 544 nm and emission at 620 nm. Data were plotted to a sigmoid curve with variable slope using Prism 5.0 (GraphPad, San Diego, CA).

2.3. Assessing cell cycle progression in T. brucei

2.3.1. DAPI staining

Nuclei and kinetoplasts were visualized using the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI) on BF trypanosomes after fixation. 50 μL of cells at ~5 × 10^5 cells/mL were spread onto a glass microscope slide, left to air dry and fixed in methanol overnight at −20 °C. The slides were rehydrated with 1 mL of PBS for 10 min, which was allowed to evaporate (but not to completely dry). 50 μL of PBS containing 1 μg/mL DAPI and 1% of 1,4-diazabicyclo [2.2.2] octane (DAPCO) was added to the slides and