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Novel inhibitors of the trypanosome alternative oxidase inhibit *Trypanosoma brucei brucei* growth and respiration

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Abstract

African trypanosomiasis is a deadly disease for which few chemotherapeutic options are available. The causative agents, *Trypanosoma brucei rhodesiense* and *T. b. gambiense*, utilize a non-cytochrome, alternative oxidase (AOX) for their cellular respiration. The absence of this enzyme in mammalian cells makes it a logical target for therapeutic agents. We designed three novel compounds, ACB41, ACD15, and ACD16, and investigated their effects on trypanosome alternative oxidase (TAO) enzymatic activity, parasite respiration, and parasite growth *in vitro*. All three compounds contain a 2-hydroxybenzoic acid moiety, analogous to that present in SHAM, and a prenyl side chain similar to that found in ubiquinol. ACD15 and ACD16 are further differentiated by the presence of a solubility-enhancing carbohydrate moiety. Kinetic studies with purified TAO show that all three compounds competitively inhibit TAO, and two compounds, ACB41 and ACD15, have inhibition constants five- and three-fold more potent than SHAM, respectively. All three compounds inhibited the respiration and growth of continuously cultured *T. b. brucei* bloodstream cells in a dose-dependent manner. None of the compounds interfered with respiration of rat liver mitochondria, nor did they inhibit the growth of a continuously cultured mammalian cell line. Collectively, the results suggest we have identified a new class of compounds that are inhibitors of TAO, have trypanocidal properties *in vitro*, and warrant further investigation *in vivo*.

Keywords: *Trypanosoma brucei brucei*; Trypanosome alternative oxidase; Inhibition kinetics; African sleeping sickness

1. Introduction

African trypanosomiasis or sleeping sickness is a major health problem that threatens over 60 million people in 36 sub-Saharan African countries (WHO, 2001). Sleeping sickness is a vector-borne, parasitic disease caused by flagellated protozoa of the genus *Trypanosoma*. The parasites are transmitted to their vertebrate hosts through bites of the tsetse fly. *Trypanosoma brucei rhodesiense* and *T. b. gambiense* cause human sleeping sickness, and *T. b. brucei* causes nagana in live-

Abbreviations: TAO, trypanosome alternative oxidase; rTAO, recombinant trypanosome alternative oxidase; AOX, alternative oxidase; SHAM, salicylhydroxamic acid; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, DL-dithiothreitol; β-NADH, β-nicotinamide adenine dinucleotide; EC_{50}, effective concentration 50; IC_{50}, inhibitory concentration 50

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stock. The parasites multiply in the bloodstream of the host and eventually invade the central nervous system, where they cause neurological disorders. The disease is fatal if left untreated (Nappi and Vass, 2002). Unfortunately, chemotherapeutic options for this disease are far from ideal. Four drugs are currently approved for the treatment of trypanosomiasis: suramin, pentamidine, melarsoprol, and eflornithine (Wang, 1995; Burchmore et al., 2002; Hoet et al., 2004; Moore, 2005). Among these, eflornithine is the only drug for which the mechanism of action is clearly documented (Bacchi et al., 1980; Burri and Brun, 2003). Collectively, this group of drugs has been plagued by problems with acute toxicity (Wang, 1995; Burchmore et al., 2002; Hoet et al., 2004). Furthermore, most of these drugs are administered by injection over long periods of time and thus, require specialized medical facilities and staff, which are frequently rare in rural areas of sub-Sahara Africa (Legros et al., 2002). The emergence of new strains of *T. brucei* with resistance to one or more of the drugs has exacerbated an already difficult situation (Legros et al., 1999; Brun et al., 2001; Burri and Keiser, 2001; Matovu et al., 2001; Moore and Richer, 2001). Despite numerous incidents of toxicity and treatment failures (Pepin and Mpia, 2005), all but one of the drugs in this collection (eflornithine) was developed over fifty years ago. There is an urgent need for new drugs against trypanosomes, that are safe, effective, and easy to administer, and have defined mechanisms of action (Cross, 2005; Moore, 2005).

The process of designing new chemotherapeutic agents for the treatment of trypanosomiasis will benefit from a better understanding of the biochemical differences between the parasite and its host. In the course of their life cycle, African trypanosomes undergo both biochemical and morphological differentiation (Vickerman, 1965; Opperdoes and Borst, 1977; Bienen et al., 1983; Vickerman, 1985; Opperdoes, 1987; Nappi and Vass, 2002). In the insect midgut, the parasite possesses a complete mitochondrion with a full complement of cytochromes. In vertebrates, the trypanosome possesses a rudimentary mitochondrion devoid of cytochromes. In this form, respiration is solely dependent on a non-cytochrome, alternative oxidase (Vickerman, 1965; Brown et al., 1973; Hill, 1976; Bienen et al., 1983; Clarkson et al., 1989; Priest and Hajduk, 1994; ter Kuile, 1994). The trypanosome alternative oxidase, or TAO, is unique to the parasite and essential for its viability. Hence, this enzyme represents an ideal target for the development of trypanocidal agents (Helfert et al., 2001; Verlinde et al., 2001). In this study we designed and synthesized a family of novel compounds using information derived from previous inhibitors and advances in our understanding of the biochemistry of alternative oxidases. Presented here is a report of the design, synthesis, and experimental screening of these novel compounds.

2. Materials and methods

2.1. Expression and purification of rTAO

Competent BL21 cells (Novagen, Madison, WI) were transformed with 100 ng of pET28a containing the cloned TAO gene and plated on LB agar supplemented with 50 μg/ml kanamycin for overnight at 37 °C. A single colony was inoculated into a 30 ml overnight culture of LB containing 50 μg/ml kanamycin and incubated at 37 °C with shaking. The next morning, 2 L of fresh LB containing 50 μg/ml kanamycin was inoculated with 12 ml of the overnight culture. The culture was shaken at 37 °C, and 1 mM IPTG was added when the O.D. reached 0.6 at 600 nm. The culture was then shaken for 4 h at 37 °C. Cells were harvested by centrifugation at 6000 rpm. The cell pellet was resuspended in 15 ml of ice-cold lysis buffer (50 mM Tris–HCl, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1% *n*-octyl-β-D-glucopyranoside, 10 mg lysozyme, 1 mM $MgCl_2$, 250 units benzonase endonuclease (Novagen), 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 5 μg/ml aprotinin). The lysate was incubated at 4 °C for 1 h with end-over-end rotation. Cell debris was collected by centrifugation at 15,000 rpm for 15 min at 4 °C. The supernatant (extract #1) was removed and stored on ice. The pellet was resuspended in 10 ml of fresh lysis buffer to extract additional protein. This material was incubated on ice for 20 min and agitated by vortexing every 5 min. Soluble protein was separated from insoluble fraction by centrifugation at 15,000 rpm for 15 min. The supernatant was saved and the pellet was extracted once more in an identical manner. The soluble fractions isolated from all three extractions were pooled and applied to 1 ml of Ni-NTA agarose (Qiagen, Valencia, CA) pre-equilibrated in PBS. The mixture was incubated at 4 °C with end-over-end rotation for 1.5 h. The resin was washed five times with 10 ml of wash buffer (50 mM Tris–HCl, 300 mM NaCl, 10% glycerol, and 1% *n*-octyl-β-D-glucopyranoside) supplemented with the following amounts of imidazole: 10 mM, 10 mM, 40 mM, 60 mM, and 80mM. The recombinant TAO was eluted from the resin in nine fractions of 1 ml each with elution buffer (50 mM Tris–HCl, 300 mM NaCl, 10% glycerol, 1% *n*-octyl-β-D-glucopyranoside, 250 mM imidazole).