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Artemisinins Inhibit *Trypanosoma cruzi* and *Trypanosoma brucei rhodesiense* In Vitro Growth[▽]

Yuliya V. Mishina,[1] Sanjeev Krishna,[2] Richard K. Haynes,[3] and John C. Meade[1]*

Department of Microbiology, University of Mississippi Medical Center, Jackson, Mississippi[1]; Division of Cellular and Molecular Medicine, Centre for Infection, St George's, University of London, Cranmer Terrace, London, United Kingdom[2]; and Department of Chemistry, The Hong Kong University of Science and Technology, Kowloon, Hong Kong, People's Republic of China[3]

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Artemisinin compounds inhibit in vitro growth of cultured *Trypanosoma cruzi* and *Trypanosoma brucei rhodesiense* at concentrations in the low micromolar range. Artemisinin also inhibits calcium-dependent ATPase activity in *T. cruzi* membranes, suggesting a mode of action via membrane pumps. Artemisinins merit further investigation as chemotherapeutic options for these pathogens.

Diseases caused by insect-borne trypanosomatid parasites are a significant and neglected public health problem worldwide. Chagas' disease, caused by infection with *Trypanosoma cruzi*, is a major agent of disease in Latin America, with 16 to 18 million infected individuals and an annual death toll of 50,000. It is also an emerging problem in the United States, where an estimated 100,000 infected individuals reside (16). *Trypanosoma brucei* subspp., the causative agents of human African trypanosomiasis or "sleeping sickness," infect 50,000 annually. Approximately 300,000 to 500,000 people have trypanosomiasis and will die if not treated (18). Current therapeutic options for *Trypanosoma* infections, benznidazole and nifurtimox for Chagas' disease treatment and suramin, pentamidine, melarsoprol, and eflornithine for treatment of sleeping sickness, are far from ideal (8, 17). These drugs all suffer from one or more disadvantages—high cost, parenteral administration, long treatment courses (months), high clinical failure rates, or parasite drug resistance—and they elicit multiple, serious, and potentially fatal toxic side effects. New therapeutic alternatives are obviously desirable for treatment of these life-threatening infections.

Artemisinin is a sesquiterpene lactone isolated from *Artemisia annua*, an annual herb that has been used in traditional Chinese medicine for over 2,000 years (21). Artemisinin is hydrophobic, passes biological membranes easily, and is a potent antimalarial with effective 50% inhibitory concentrations (IC_{50} values) ranging from 4.2 to 16.2 nM for different derivatives. Oral, parenteral, or rectal dosages achieve micromolar plasma concentrations (22). Artemisinin derivatives have been used to treat malaria cases around the world, and their extensive usage has not been associated with any significant toxicity (19). Artemisinin generates bioreactive radicals capable of intracellular damage, depolarizes mitochondrial membrane potential in yeast, and inhibits the *Plasmodium falciparum* endoplasmic reticulum calcium pump (SERCA), and artemisinin-resistant *P. falciparum* contains SERCA mutations (9, 13, 14, 20–22).

Artemisinin compounds also show efficacy against *Leishmania* spp. of trypanosomatid parasites, achieving 50% killing at 750 nM for *Leishmania major* promastigotes, at 3 to 30 μM for intracellular amastigote stages in macrophages, and at 1.4 to 382.9 μM against *Leishmania infantum* promastigotes (1, 23). Artemether treatment (50 mg/kg of body weight/day) of footpad lesions in mice, by oral, intralesional, intramuscular, or intravenous administration, significantly reduces lesion size and *L. major* parasite numbers (23). Oral dihydroartemisinin (25 or 50 mg/kg) also reduces parasite burdens by 75% in the spleens and livers of hamsters infected with *Leishmania donovani* (15). To date, the effects of artemisinins on *Trypanosoma* spp. have not been reported.

The effects of artemisinin, of two artemisinin derivatives, artemisone and 4-fluorophenyl artemisinin, and of dihydroartemisinin, an active metabolite produced from artemisinin derivatives within the body, on the in vitro growth of *T. cruzi* epimastigotes (3), *T. brucei rhodesiense* trypomastigotes (12), and *L. donovani* promastigotes (2) are shown in Table 1. The artemisinins each inhibited 50% of growth (IC_{50}) at low micromolar concentrations, 13.4 μM to 23.3 μM for *T. cruzi* and 15.7 μM to 22.5 μM for *T. brucei rhodesiense*. Artemisinins inhibited 90% of parasite growth at 46.5 μM to 50.8 μM for *T. cruzi* and 44.8 μM to 49.6 μM for *T. brucei rhodesiense*. The IC_{50} values for the trypanosomes are comparable to those obtained with *L. donovani* promastigotes, which have previously been shown to be susceptible to artemisinin compounds in vitro and in vivo (1, 15, 23). Similar results were also obtained in drug IC_{50} determinations for *Leishmania* and *Trypanosoma* spp. by using Alamar Blue (Biosource, Camarillo, CA), a colorimetric indicator of cell viability and proliferation (data not shown). The antitrypanosomal drug pentamidine and antileishmanial drug amphotericin B exhibited more potent efficacy than the artemisinin compounds in this assay, in which IC_{50}s and IC_{90}s were achieved at concentrations as much as 20-fold less for pentamidine and 8-fold less for amphotericin (Table 1). However, these two drugs were much more toxic to a human cell line than artemisinin. The growth of human U-937 monocytes (ATCC, Manassas, VA) was completely inhibited by amphotericin and pentamidine at concen-

* Corresponding author. Mailing address: Department of Microbiology, University of Mississippi Medical Center, 2500 North State Street, Jackson, MI 39216. Phone: (601) 984-1914. Fax: (601) 984-1708. E-mail: jmeade@microbio.umsmed.edu.

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TABLE 1. In vitro growth inhibition of *Trypanosoma* sp. culture forms by artemisinin compounds[a]

Drug	*T. cruzi*		*T. brucei rhodesiense*		*L. donovani*
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}
Artemisinin	13.4 ± 2.3	46.5 ± 1.2	20.4 ± 0.3	49.1 ± 1.3	30.8 ± 1.4
Artemisone	23.3 ± 2.7	50.5 ± 0.8	22.5 ± 3.0	44.8 ± 0.8	12.9 ± 6.3
4-Fluorophenyl-artemisinin	17.9 ± 3.9	50.8 ± 0.9	15.7 ± 5.6	46.9 ± 0.6	3.0 ± 1.1
Dihydroartemisinin	12.8 ± 0.3	49.2 ± 1.5	24.6 ± 2.4	49.6 ± 1.0	NT
Thapsigargin	33.9 ± 2.6	78.1 ± 1.6	30.1 ± 1.6	71.9 ± 1.2	28.1 ± 1.2
Pentamidine	1.1 ± 0.2	4.4 ± 0.2	1.2 ± 0.3	3.7 ± 0.2	1.0 ± 0.1
Amphotericin B	6.5 ± 1.6	19.6 ± 0.7	2.8 ± 0.7	11.9 ± 0.4	2.8 ± 0.1
Omeprazole	177.8 ± 17.3	526.2 ± 14.2	177.1 ± 31.0	509.0 ± 13.6	339.9 ± 15.9
Ouabain	>450	953.3 ± 8.4	>450	1184.0 ± 18.9	>450

[a] IC_{50} and IC_{90} values are expressed in micromolar concentrations ± standard errors of the means for parasite growth inhibition after 72 h of drug exposure. Growth was measured at an optical density at 600 nm versus that of control cultures without drug. Assays were performed two to five times with six drug concentrations, the mean inhibitions were calculated and plotted against the drug concentrations, and IC_{50} and IC_{90} values were determined by linear regression. NT, not tested.

trations of 24 μM and 12 μM, respectively (data not shown), levels which are only twofold to threefold greater than the IC_{90} values for parasite inhibition reported in Table 1. Conversely, artemisinin inhibition of U-937 growth was less than 5%, even at a 3 mM concentration of the drug, a level >60-fold above that for the parasite IC_{90} values.

Since one target for artemisinin activity in *Plasmodium*, its SERCA pump, is a member of the P-type ATPase ion pump family, additional inhibitors of P-type ATPases were also tested for their antitrypanosomal efficacy. Omeprazole, an inhibitor of the gastric H^+/K^+-ATPase, and ouabain, which inhibits cardiac Na^+/K^+-ATPase activity, are in current clinical usage, and thapsigargin is a SERCA pump inhibitor with chemical similarity to artemisinin. Thapsigargin inhibited parasites at concentrations comparable to those obtained with artemisinin compounds, while ouabain and omeprazole were much less inhibitory to parasite growth (Table 1).

Artemisinin could also substitute for the classic SERCA inhibitor thapsigargin in the inhibition of calcium-dependent ATP hydrolysis in *T. cruzi* membrane preparations. *T. cruzi* membranes were prepared using a previously described protocol for isolation of membranes from *Leishmania*, a closely related trypanosomatid (6). Calcium-dependent ATPase activity was assayed by monitoring the release of inorganic phosphate from ATP in 50-μl reaction mixtures containing 50 mM MES (2-morpholinoethanesulfonic acid; pH 6.5), 5 mM $MgSO_4$, 50 mM KNO_3, 5 mM NaN_3, 2 mM sodium molybdate, 10 μM $CaCl_2$, and 10 μg of membranes. Reactions were initiated by the addition of 2 mM ATP, and the production of free phosphate was assessed using the colorimetric protocol of Chifflet et al. (4). *T. cruzi* membrane ATPase activity in the presence of calcium was inhibited 24.9% ± 1.5% by the addition of 1 μM artemisinin to the assay and 25.8% ± 3.9% by 1 μM thapsigargin. Although the *T. cruzi* SERCA pump is thapsigargin insensitive when expressed in yeast, there are three additional calcium ATPases present in the *T. cruzi* genome, and thapsigargin-sensitive calcium stores in *T. cruzi* have been documented (5, 7, 10). These results suggest that artemisinin inhibition of trypanosomes may occur, at least in part, through inhibition of a membrane-associated calcium-dependent ATPase pump.

Data presented here demonstrate that artemisinin compounds are effective against *T. cruzi* and *T. brucei rhodesiense* parasites at micromolar concentrations that are close to those clinically achievable with current artemisinin-based therapies. *T. brucei* trypomastigotes circulate in the bloodstream during infection, and *T. cruzi* forms are also present in the blood during the acute stage of infection, and so both parasites are readily accessible to the action of artemisinin drugs. In view of the current, considerable investment in the development and characterization of new artemisinin compounds, this finding offers unique opportunities to exploit artemisinins as therapeutic agents for these life-threatening pathogens. The ongoing development of artemisinin compounds with improved safety and physiochemical profiles relative to the current artemisinin antimalarials, such as the artemisone compound tested here, supports that concept (11). In addition, confirmation of *Trypanosoma* calcium pumps as a target for artemisinin would also facilitate future development of antitrypanosomal artemisinin compounds by enabling molecular modeling of their interaction with target biomolecules. Thus, artemisinin compounds merit consideration and further investigation as therapeutic options in the treatment of human trypanosomiasis.

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