

In Vitro Inhibition of *Toxoplasma gondii* by Four New Derivatives of Artemisinin[∇]

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Toxoplasmosis, caused by the protozoan *Toxoplasma gondii*, is medically important and distributed worldwide. Currently available medications are limited in terms of efficacy and side effects. We synthesized novel, nonacetal, hydrolytically stable derivatives of artemisinin and showed that they inhibit the replication of *Toxoplasma gondii* in cell culture.

Toxoplasma gondii is an apicomplexan protozoan of worldwide medical importance. Humans are infected by *T. gondii* through contact with feces from infected cats, by the consumption of undercooked meat from infected animals, or by transmission from infected mother to fetus. This parasite can cause systemic infection and widespread organ damage in immunocompromised individuals and neonates. Infection of immunocompetent adults can result in fever and adenopathy (11). Serological studies indicate that *T. gondii* could be associated with chronic neuropsychiatric diseases or behavioral abnormalities in some populations (1, 12).

Available medications for the prevention and treatment of toxoplasma infection show limited efficacy and have substantial side effects (5). Published studies have indicated that the naturally occurring 1,2,4-trioxane artemisinin and artemisinin derivatives such as artemether, originally developed for the treatment of malaria, have the ability to inhibit toxoplasma replication in vitro (2, 4, 6, 10). While these trioxanes have a number of advantages in terms of rapid action and low levels of toxicity, they are limited in terms of absorption, bioavailability, and short half-life (i.e., easy hydrolysis into toxic dihydroartemisinin) (9). Thus, we have designed and synthesized five C-10 nonacetal, hydrolytically stable derivatives of artemisinin (8) and measured their ability to inhibit the replication of *T. gondii* in vitro.

Artemisinin was obtained from Holley Pharmaceuticals Co., Inc., Fullerton, CA. Artemether was generously donated by Huiling Wang, University of Wuhan, China. Trimethoprim was purchased from Sigma Chemical Co. (St. Louis, MO). Chlorophenol red- β -D-galactopyranoside (Roche, Indianapolis, IN), 100 mM in 100 mM HEPES (pH 7.2), was stored frozen at -80°C . CellTiter 96 AQ_{ueous} One Solution Reagent for determining cytotoxicity was purchased from Promega Corp., Madison, WI.

Structures of artemisinin and the derivatives are shown in Fig. 1. Trioxane C-10 primary alcohol 2a was prepared in 74% yield by hydroboration-oxidation of the corresponding known C-10 allyl trioxane. Esterification of primary alcohol 2a with benzoyl chloride led to benzoate ester 2b in 92% yield, and esterification with 2-bromobenzoyl chloride produced 2-bromobenzoate ester 2c in 86% yield. Deprotonation of the primary alcohol 2a using sodium hydride and then displacement of the bromide anion from citronellyl bromide gave citronellyl ether 2d in 55% yield. First, zinc-promoted deoxygenation of the known C-10 allyl trioxane and then hydroboration-oxidation produced dioxolane deoxy-2a in 72% yield. Each of these new lipid-soluble chromatographically purified artemisinin derivatives was fully characterized spectroscopically (proton and carbon-13 nuclear magnetic resonance, infrared, and high-resolution mass spectrometry). In contrast to artemether, these trioxane monomers are hydrolytically stable for at least 12 h even at 60°C in dimethyl sulfoxide-water at pH 7.4. Under these conditions, less than 5% decomposition was observed by proton nuclear magnetic resonance spectrometry.

Normal human foreskin fibroblasts (American Type Culture Collection, Manassas, VA) were used to grow tachyzoites and to test compounds for activity and cytotoxicity. Cells were maintained in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 25 mM HEPES (Gibco), 2 mM L-glutamine, 50 units of penicillin G per ml, and 50 μg of streptomycin sulfate per ml. A culture of the tachyzoites of *Toxoplasma gondii* strain 2F, which constitutively expresses cytoplasmic β -galactosidase and is derived from strain RH, was a gift from Vern Carruthers, University of Michigan Medical School.

Compounds were tested for in vitro efficacy against *T. gondii* and cytotoxicity by previously published methods (7). The median inhibitory dose (50% inhibitory dose [ID₅₀]) and the median cytotoxic dose (50% toxic dose [TD₅₀]) were calculated by extrapolation of the corresponding dose-response curve on a log-linear plot employing the portions of the curve that transected the 50% response point. For each compound, a therapeutic index (TI) was calculated by the formula $\text{TI} = \text{TD}_{50}/\text{ID}_{50}$.

The results of the toxoplasma inhibition and cytotoxicity

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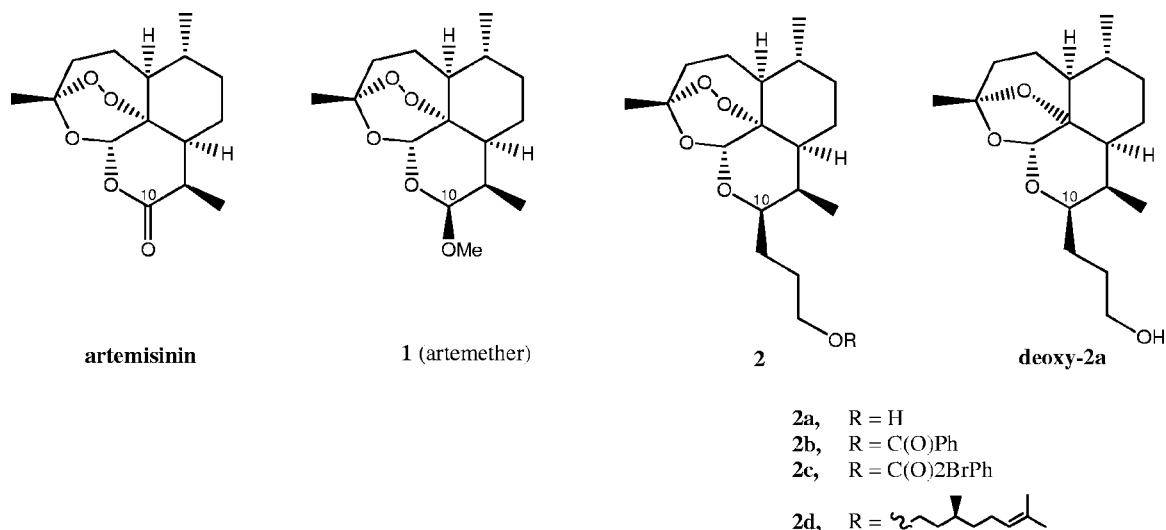


FIG. 1. Structures of artemisinin, artemether, and novel derivatives. Substitutions at R are as shown.

assays are shown in Table 1. Artemether, 2b, 2c, and 2d all inhibited the toxoplasma at concentrations of less than 1 $\mu\text{g}/\text{ml}$. Somewhat less antitoxoplasma activity was noted with artemisinin and compound 2a, which inhibited toxoplasma replication at concentrations between 2 and 3 $\mu\text{g}/\text{ml}$. These values compare favorably to that of the antifolate positive-control compound trimethoprim, which inhibited toxoplasma replication at a concentration of 5.2 $\mu\text{g}/\text{ml}$. Nonperoxidic deoxy-2a showed virtually no inhibitory activity against the toxoplasma, with a TI of 1.2.

In order to determine therapeutic indices and thus specific antitoxoplasma activity, we also measured the cytotoxicity of the test compounds. Artemisinin, 2a, 2b, and 2c showed little cytotoxicity at concentrations up to 510 $\mu\text{g}/\text{ml}$ (compound 2a) or 320 $\mu\text{g}/\text{ml}$ while the other artemisinin derivatives showed 50% cytotoxicity only at concentrations of >160 $\mu\text{g}/\text{ml}$. These degrees of cytotoxicity compare favorably with that of trimethoprim, with a TD_{50} of 60 $\mu\text{g}/\text{ml}$. The therapeutic indices of all of the compounds except deoxy-2a were thus approximately 10 \times to 100 \times more favorable than that of trimethoprim.

All of the compounds that showed inhibitory activity in our

assays towards *T. gondii* have been shown to inhibit the replication of chloroquine-sensitive *Plasmodium falciparum* (NF 54) strains of malaria with ID_{50} s from 5 to 30 nM (J. D'Angelo and G. Posner, unpublished data). And further, as expected (3), nonperoxidic deoxy-2a, which has virtually no antimalaria activity (J. D'Angelo and G. Posner, unpublished data), was also devoid of antitoxoplasma activity. These findings suggest that derivatives of artemisinin may affect similar pathways in toxoplasma and malarial organisms. The elucidation of these shared pathways should be the subject of additional investigations.

We have demonstrated that four new nonacetal derivatives of artemisinin have both increased antitoxoplasma activity and decreased cytotoxicity compared to trimethoprim, one of the antifolate compounds of relatively low toxicity that is used for the treatment of toxoplasma infection in humans. While other antifolate compounds, such as pyrimethamine, have increased antitoxoplasma activity, their toxicity generally precludes widespread usage, particularly over prolonged periods of administration (5). The availability of low-toxicity compounds capable of the prevention and treatment of *T. gondii* in humans would represent a major advance in the treatment of infections in immunocompromised individuals. Further, the availability of such compounds would also allow for clinical trials directed at defining the role of toxoplasma infections in human diseases.

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TABLE 1. In vitro inhibition of *T. gondii* by artemisinin and synthetic derivatives

Compound ^a	Mol wt	ID_{50}		TD_{50}		TI
		$\mu\text{g}/\text{ml}$	μM	$\mu\text{g}/\text{ml}$	μM	
Artemisinin	282.34	2.3	8.0	>320 ^b	>1,130	\geq 243
Artemether	298.4	0.2	0.7	220	740	1,100
2d	464.68	0.5	1.1	160	340	320
2c	509.43	0.6	1.2	>320 ^b	>630	\geq 933
2b	430.54	0.6	1.4	>320 ^b	>740	\geq 933
2a	326.43	2.7	8.3	510	1,560	190
Deoxy-2a	310.43	173	560	200	640	1.2
Trimethoprim	290.3	5.2	17.9	60	210	12

^a The parent compound is shown first followed by the known derivative. The five new derivatives are listed in decreasing order of efficacy according to concentrations in $\mu\text{g}/\text{ml}$.

^b Toxicity endpoint was not reached; a value 1/4 log greater than the concentration tested was used to compute the TI.

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