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Inhibition of Growth of *Toxoplasma gondii* by Qinghaosu and Derivatives

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The antimalarial compound qinghaosu (artemisinin) was tested in vitro for the ability to inhibit plaque formation by *Toxoplasma gondii* in fibroblasts. Qinghaosu at 0.4 μg/ml for 5 days eliminated all plaques and microscopic foci of *T. gondii*. At 1.3 μg/ml for 14 days, qinghaosu completely eliminated *T. gondii*. Pretreatment of host cells or *T. gondii* with qinghaosu had no effect on *T. gondii* growth. There was no apparent toxicity to human fibroblasts in long-term studies. Of the six qinghaosu derivatives tested, dihydroqinghaosu, 1-propyl-ether-qinghaosu, and 1-butyl-ether-qinghaosu were comparable to qinghaosu. Ethyl-ether-qinghaosu (arteether) and sec-butyl-ether-qinghaosu were more effective. Methyl-ether-qinghaosu (artemether) was the most effective, with a potency approximately 10-fold greater than that of qinghaosu.

The Chinese herb, qing hao (*Artemisia annua* L.), is the source of a recently isolated effective antimalarial compound artemisinin or qinghaosu (QHS; translated as green herb compound). The use of an aqueous infusion of qing hao to treat the fever of malaria dates back to Zhou Hou Bei Ji Fang (*Handbook of Prescriptions for Emergency Treatment*) written in 340 A.D. by Ge Hong. Only in 1972 was the active constituent of qinghao, a sesquiterpenelactone peroxide, isolated (Fig. 1) (11, 24). Subsequently, a number of derivatives of QHS have been synthesized, some of which are more effective antimalarial agents than the parent compound (see reference 16 for a recent review of QHS). The toxicity of QHS and several derivatives is quite low, with chemotherapeutic indices for malaria of over 2,000 (8).

Toxoplasma gondii is an obligate intracellular protozoal parasite distributed worldwide in tropical and milder temperate climates. Its definitive host is the feline, but it is capable of infecting all animals tested. In healthy mammals *T. gondii* produces a chronic asymptomatic infection except if acquired during pregnancy, in which case it frequently produces moderate to severe fetal damage (25). With the advent of immunosuppressive therapy and acquired immunodeficiency syndrome latent infections are reactivating and presently *T. gondii* is the most common cause of focal encephalitis in patients with acquired immunodeficiency syndrome (13).

The present antitoxoplasmic drugs are commonly poorly tolerated by patients with acquired immunodeficiency syndrome (13), and effective alternative drugs are needed. Novel compounds effective against *Plasmodium* species may be effective against *Toxoplasma* species since the genera are related (20). We report here the results of studies of the antitoxoplasmic activities of QHS and six derivatives (Fig. 1).

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MATERIALS AND METHODS

Growth of tissue cultures and organisms. The RH strain of *T. gondii*, obtained from J. D. Schwartzman (originally from E. R. Pfefferkorn), was maintained in locally obtained human embryonic lung fibroblasts of less than 30 passages. The fibroblasts were grown in VA-13 medium (minimal essential medium (MEM) with Earle salts, 2× MEM essential amino acids, 1× MEM nonessential amino acids, 2× MEM vitamins, 3× glucose, 1 mM sodium pyruvate, 100 U of penicillin G per ml, 100 μg of streptomycin sulfate per ml), pH 7.2. VA-13 medium was supplemented with 10% adult bovine serum for nonparasitized fibroblasts. For growth of *T. gondii* in stationary-phase fibroblasts, VA-13 was used with 0.3% bovine serum albumin (Cohn fraction V). When both the fibroblasts and *T. gondii* were grown concurrently, the VA-13 was supplemented with fetal bovine serum (10%). All cultures were maintained in 5% CO_2–95% air at 37°C.

Quantitation of QHS. QHS (artemisinin), dihydro-QHS (dihydroartemisinin), methyl-ether-QHS (artemether), ethyl-ether-QHS (arteether), 1-propyl-ether-QHS, 1-butyl-ether-QHS, and sec-butyl-ether-QHS were obtained from Hauser Chemical Research, Inc. (Boulder, Colo.). Stock solutions were prepared in DMSO (dimethyl sulfoxide). The concentration of QHS was verified by using the method of Zhao and Zeng (28). Briefly, 1 part of QHS solution was mixed with 4 parts of 50 mM NaOH and heated for 30 min at 45°C. The mixture was rapidly cooled to room temperature, and 5 parts of 100 mM acetic acid in 20% ethanol was added. The final reaction mixture was diluted 1/20 with 20 mM potassium phosphate buffer at pH 6.0, and the optical density was measured at 259 nm with a Beckman DU-7 spectrophotometer. The blank used for the spectrophotometer was the DMSO solvent processed in parallel. The extinction coefficient used was $1.3 \times 10^4\ M^{-1}\ cm^{-1}$ (28).

The QHS derivatives were formulated on a weight-to-volume basis.

Five-day plaque assay. The 5-day plaque assay is based on the method of Chaparas and Schlesinger (2) as modified by

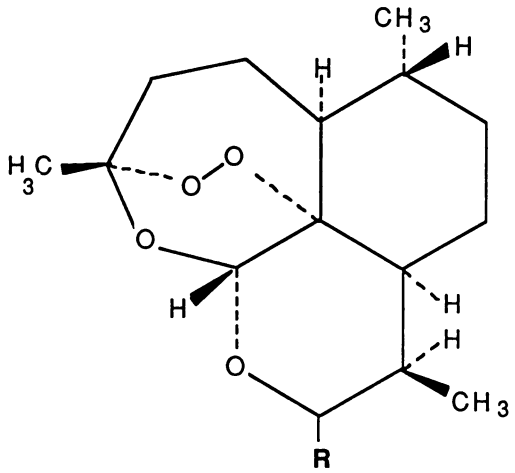

FIG. 1. Structure of qinghaosu and derivatives. Substitutions at **R** gave the following derivatives: O, qinghaosu; OH, dihydro-QHS; OCH_3, artemether (methyl-ether-QHS); OCH_2CH_3, arteether (ethyl-ether-QHS); $OCH_2CH_2CH_3$, 1-propyl-ether-QHS; $OCH_2CH_2CH_2CH_3$, 1-butyl-ether-QHS; $OCHCH_3CH_2CH_3$, sec-butyl-ether-QHS.

Pfefferkorn and Pfefferkorn (23). Fibroblasts were inoculated into 12-well culture plates and grown to confluence. Within 3 days after reaching confluence, the monolayers were infected with 200 *T. gondii* parasites per well (1 parasite per 1,000 fibroblasts). Unless otherwise indicated, the parasites were incubated with the fibroblasts for 3 h before addition of the drug. In each assay, two parasitized culture wells per group received no drug, pyrimethamine (10 μg/ml) as a positive control (9), QHS, or DMSO at the concentrations in the matched QHS wells. The culture plates were incubated for 5 days in a humidified chamber under standard culture conditions without movement so that distinct plaques were formed. The cultures were then examined by phase-contrast microscopy to assess the condition of the fibroblasts and the *T. gondii*. The old medium was decanted, the cultures were fixed with methanol for 5 min and air dried, and the plaques were counted with the aid of an American Optical dark-field Quebec colony counter. The plaque size was quantitated on a "four-plus" scale. Four plus is the size of the control plaques. Three plus is a plaque diminished in size but larger than half size, which is two plus. One plus is a barely visible plaque.

For microscopic visualization of fixed *T. gondii*, the fixed fibroblast monolayers were stained for 30 min with a 1:50 dilution of Giemsa stain in distilled water (Harleco, Gibbstown, N.J.).

Fibroblast preincubation with QHS. Fibroblast culture plates were grown to confluence. Then, one culture plate received QHS in the appropriate wells for each of 5 consecutive days. After 5 days, the QHS was removed and 200 *T. gondii* parasites were added per well. After 5 additional days, the results were scored as described above.

***T. gondii* preincubation with QHS.** From a population of free fresh *T. gondii*, 200 parasites were added per well to three culture wells containing fibroblasts. These served as time zero controls. The remaining parasites were divided into five groups and placed in VA-13 with 0.3% albumin under standard culture conditions. One group each received QHS at 4 or 0.4 μg/ml, DMSO at 0.1 or 0.01%, or no drug. At 2 and 4 h, 200 parasites and at 16 h, 400 parasites from each group were added to fibroblast cultures. This form of *T. gondii* gradually dies since it remains outside host cells. Therefore, a larger number of parasites (400) were used of those maintained with and without drug for 16 h. One hundred-twenty hours after the addition of *T. gondii*, the cultures were terminated and quantitated as described above.

TABLE 1. Pretreatment of fibroblasts[a]

Time (h)	Pretreatment (% of control)	
	DMSO	QHS
24	114	89
48	104	90
72	109	100
96	99	91
120	102	100

[a] QHS was present at 0.4 μg/ml; DMSO was present at 0.01%.

Effectiveness of QHS derivatives on *T. gondii*. The qinghaosu derivatives were tested as described above.

Long-term studies. A fibroblast monolayer in a 150-cm^2 culture flask was infected with 6,400 *T. gondii* parasites (1/5,000 fibroblasts). After 3 days, the infected fibroblasts were detached by trypsin treatment and subcultured at one-fourth the cell density in 25-cm^2 culture flasks. The infected fibroblasts were allowed to adhere overnight before they received any drug. Time zero was defined as the time of drug addition. Duplicate flasks were used for each treatment group. Five experimental groups were used. Three groups received QHS at 0.4, 1.3, or 4.0 μg/ml. One group received drug-free culture medium (infected controls), and one group (positive drug controls) received pyrimethamine at 10 μg/ml.

Drug-treated cultures were grown for 5 days and then subcultured at a 1:4 dilution. One subcultured flask continued with uninterrupted drug exposure while a second flask was allowed to grow drug free. The cultures that continued to receive drug exposure were grown to confluence and then subcultured into two groups as described above. This process was repeated until the flasks of that drug concentration were terminated as described below. Infected controls were subcultured at a 1:4 dilution until death.

The subcultures that ceased receiving drug were repeatedly subcultured when confluent. Cultures were considered *T. gondii* free if they could be subcultured four times without drug with no evidence of infection. In the event that a flask containing drug-free medium displayed *T. gondii* growth, it was terminated.

Materials. Fetal bovine serum was obtained from Hyclone (Logan, Utah), and plasticware was from Corning (Littleton, Colo.). All other culture media were from Sigma Chemical Co. (St. Louis, Mo.). All reagents were of analytical grade.

RESULTS

Effects of QHS on *T. gondii* growth. QHS at 0.23 μg/ml allowed growth of only 7 to 10 parasites per infection site, yielding insufficient damage to produce a macroscopically visible plaque. At higher concentrations, neither plaques nor sites of *T. gondii* growth were found. At 23 μg/ml, the highest concentration tested, the morphology of the fibroblasts appeared normal. DMSO did not stop plaque

TABLE 2. Effects of QHS and derivatives on *T. gondii* plaques

Drug	Growth[a] (% of DMSO control) at drug concn of:			
	10 μg/ml	1 μg/ml	0.1 μg/ml	0.01 μg/ml
QHS	0	0	89 (++)	104 (++++)
Dihydro-QHS	0	0	76 (++)	101 (++++)
Methyl-ether-QHS	0	0	0	98 (+)
Ethyl-ether-QHS	0	0	*	100 (++++)
1-Propyl-ether-QHS	0	0	84 (+)	109 (++++)
1-Butyl-ether-QHS	0	0	98 (++)	100 (++++)
Sec-butyl-ether-QHS	0	0	*	92 (++++)

[a] Sizes of plaques are expressed on a four-plus scale. ++++, Normal-size plaque; ++, half-size plaque; +, barely visible plaque; *, some *T. gondii* seen upon microscopy.

TABLE 3. Elimination of *T. gondii* from fibroblasts in long-term studies

Treatment	Elimination[a]				
	Day 0	Day 5	Day 14	Day 22	Day 27
QHS					
0 μg/ml	Δ	Δ	×		
0.4 μg/ml	Δ	Δ	×		
1.3 μg/ml	Δ	Δ	Δ	0	0
4 μg/ml	Δ	Δ	0	0	0
Pyrimethamine (10 μg/ml)	Δ	Δ	0	0	0

[a] Δ, *T. gondii* present; 0, no *T. gondii* present; ×, host cells destroyed.

formation even at the highest concentrations used (0.6%). However, it was found that DMSO concentrations above 1.6% interfered with the plaque assay and at 10% caused release of intracellular *T. gondii* within 15 min. Pyrimethamine at 0.1 to 10 μg/ml eliminated all signs of *T. gondii* infection.

Effect of pretreatment of fibroblasts and *T. gondii*. The influence of pretreatment of fibroblasts with QHS or DMSO is presented in Table 1. Pretreatment of fibroblasts for 120 h with QHS had no effect on their ability to support growth of *T. gondii*.

T. gondii parasites were pretreated with QHS at 4 and 0.4 μg/ml. The concentrations were chosen to greatly exceed (4 μg/ml) or slightly exceed (0.4 μg/ml) the effective concentrations of QHS. Pretreatment with QHS in all cases had no detectable effect on *T. gondii*.

Effectiveness of QHS derivatives on *T. gondii*. The relative effectiveness of QHS and six derivatives is presented in Table 2. At 1 μg/ml, all compounds prevented macro- and microscopic evidence of parasite growth. At 0.1 μg/ml, all compounds reduced the size of plaques formed and all but 1-butyl-ether-QHS also reduced the plaque number. Methyl-ether-QHS eliminated all evidence of growth at this concentration. Ethyl-ether-QHS and sec-butyl-ether-QHS permitted growth evident only at the microscopic level. At 0.01 μg/ml, only methyl-ether-QHS inhibited *T. gondii* growth as evidenced by the diminished plaque size.

Elimination of *T. gondii* from fibroblast cultures by QHS in long-term studies. The results of long-term incubation of infected fibroblasts with QHS are presented in Table 3. By 14 days, all untreated fibroblasts and those treated with qinghaosu at 0.4 μg/ml were destroyed. Qinghaosu at 1.3 μg/ml reduced *T. gondii* plaques from an uncountable number at 5 days to 13 plaques per 25-cm^2 culture after 14 days. Treatment for 22 days eliminated the infection. Qinghaosu treatment at 4.0 μg/ml eliminated *T. gondii* by 14 days. The morphology and growth of the uninfected drug control fibroblasts was normal.

Pyrimethamine at 10 μg/ml eliminated *T. gondii* after 14 days. Fibroblasts treated with pyrimethamine at this concentration grew slowly and by 14 days shed irregular refractile fragments. Similar growth inhibition was observed when pyrimethamine was used at the lower concentration of 1 μg/ml (data not shown).

Effects of host cell growth conditions on QHS killing of *T. gondii*. The concentration of QHS necessary to completely eliminate *T. gondii* from fibroblast cultures in long-term studies (1.3 μg/ml [Table 3]) was higher than that necessary to prevent plaque formation (0.4 μg/ml) in 5-day cultures. The long-term fibroblast cultures differ in at least two ways from the plaque assays. First, they contain actively growing fibroblasts rather than stationary-phase fibroblasts; second, the incubation medium contains 10% serum instead of 0.3% albumin. These differences may have caused the change in effectiveness of QHS.

To determine the effects of serum, confluent monolayers of fibroblasts received either 0.3% albumin or 10% fetal calf serum and were then infected with *T. gondii* at 200 parasites per well. Within 3 h, the appropriate drugs were added, and after 5 days the cultures and plaques were quantified as above. The serum reduced the apparent effectiveness of QHS at the marginal concentration of 0.1 μg/ml and resulted in larger plaques (++ versus +) compared with the 0.3% albumin-based medium. The concentration that eliminated plaques was identical for both formulations. Plaque sizes were identical in drug-free cultures for both medium formulations. The use of 10% serum in place of 0.3% albumin resulted in a 25% reduction in plaque number in all cases.

To compare the effectiveness of QHS on parasite growth in actively dividing versus nondividing host cells, confluent fibroblasts were subcultured at a 1:5 dilution in culture wells. Three hours later, these wells and culture wells containing confluent, nondividing fibroblasts received 200 *T. gondii* parasites per well, and the appropriate drugs were added in 10% serum. The cultures were incubated for 5 days and then examined as above. Growth status of the host cells did not alter effectiveness of QHS since visible parasite growth was stopped by QHS at 1 μg/ml in both growing and nongrowing fibroblast cultures.

DISCUSSION

The effectiveness and low toxicity of qinghaosu against malaria are well established (3, 5–8, 14, 24). Clinical trials of QHS and its derivatives against malaria are presently being conducted by the World Health Organization and the U.S. Department of Defense. In addition to the inhibitory effects of QHS on the asexual erythrocytic stage of malaria, QHS and its derivatives have been reported to be partially effective against *Schistosoma mansoni*, *Schistosoma japonica* (18, 19, 26, 27), and an unspecified schistosome (21). The trematode *Clonorchis sinensis* (4) and the ameba *Naegleria fowleri* (10) were likewise susceptible. Gram-positive and gram-negative bacteria tested were not inhibited by QHS (17).

Our results show that *T. gondii* growth is inhibited by this drug. Pretreatment of fibroblasts or *T. gondii* with QHS had no effect.

Structure-activity studies have shown that the labile per-

oxide bond is essential for antimalarial activity (5, 7, 15) and presumably for antitoxoplasmal activity. Modification of the hemiacetal oxygen of the δ-lactone ring has produced effective antimalarial derivatives (Fig. 1). As antimalarial agents, dihydroqinghaosu and methyl-ether-QHS (artemether) are 100 times more inhibitory to *Plasmodium falciparum* than QHS in vitro (22) and in vivo in mice (6, 15). In *Plasmodium berghei*- and *Plasmodium cynomolgia*-infected mice, methyl-ether-QHS is only 4 times more effective than QHS (6). Unfortunately, methyl-ether-QHS is also more toxic than QHS in mice (8).

Our studies have shown that methyl-ether-QHS is at least 10-fold more potent than QHS, dihydroqinghaosu, 1-propyl-ether-QHS, or 1-butyl-ether-QHS against *T. gondii* (Table 3). Ethyl-ether-QHS and sec-butyl-ether-QHS are intermediate in potency. Ethyl-ether-QHS has been tested previously in vitro and in vivo against *T. gondii* (1), with inconsistent results.

In the long-term studies, the criteria for complete clearance of *T. gondii* infection were more stringent, as one surviving parasite ultimately can produce visible plaques. Elimination of *T. gondii* by QHS was achieved with QHS at ≥1.3 μg/ml. Higher QHS concentrations reduced the treatment time.

The presence of 10% serum in place of 0.3% albumin reduced the plaque number by 25% in the short-term plaque studies and reduced the efficiency of QHS in the short- and long-term studies. The reduction in plaque number by serum has been previously reported by Doran (12), who reported that serum is inhibitory to *T. gondii* penetration of host cells. The apparent difference in QHS effectiveness between albumin and serum-based media may be due to its tendency to bind to serum proteins. Binding of the artemether derivative of QHS to serum protein has been reported to be 58% in mice, 61% in monkeys, and 77% in humans (6, 7).

QHS and its derivatives are a family of compounds that have a history of use in humans as effective antimalarial agents. The data we present here suggest that they may be useful as alternatives to antifolates for treating toxoplasmosis.

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