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In Vitro Effects of Artemisinin Ether, Cycloguanil Hydrochloride (Alone and in Combination with Sulfadiazine), Quinine Sulfate, Mefloquine, Primaquine Phosphate, Trifluoperazine Hydrochloride, and Verapamil on *Toxoplasma gondii*

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The in vitro effects of the following antimicrobial agents on *Toxoplasma gondii* tachyzoites were studied: artemisinin ether (arteether), cycloguanil hydrochloride (cycloguanil), mefloquine, primaquine phosphate, and quinine sulfate, as well as the calcium channel blocker verapamil and the calmodulin inhibitor trifluoperazine hydrochloride. Arteether at ≥0.5 μg/ml and cycloguanil at ≥1.0 μg/ml inhibited *T. gondii* in vitro. Cycloguanil (2.5 μg/ml) combined with a noninhibitory concentration of sulfadiazine (25 μg/ml) inhibited *T. gondii* more than cycloguanil alone. Neither primaquine phosphate, mefloquine, nor quinine sulfate had an inhibitory effect on intracellular *T. gondii*. Verapamil and trifluoperazine hydrochloride were not inhibitory at lower physiologic concentrations, but higher physiologic concentrations were toxic to cell cultures in vitro and therefore our assay could not be used to assess their effects.

Infection with *Toxoplasma gondii* causes significant morbidity and mortality in immunocompromised individuals and infants born to women who were acutely infected while pregnant. Currently, *Toxoplasma* encephalitis in immunocompromised patients and congenital toxoplasmosis are treated with the synergistic combination of pyrimethamine and sulfadiazine or triple sulfonamides. Since toxic effects from this therapy include bone marrow suppression and allergy to sulfonamides (especially in AIDS patients), there is currently a great need for alternative, effective antimicrobial agents. Therefore, we evaluated in vitro several antimalarial agents, as well as the calcium channel blocker verapamil and the phenothiazine trifluoperazine hydrochloride by using our previously described assay (12).

These agents were selected because malaria and *T. gondii* infections are caused by related apicomplexa protozoa and a number of antimicrobial agents effective in the treatment of malaria have also been effective in the treatment of toxoplasmosis (14). Artemisinin (qinghaosu) and its analogs artemisinin ethyl ether (arteether) and artemisinin methyl ether (artemether) are sesquiterpene lactones which are extremely effective against chloroquine-resistant malaria, especially cerebral malaria (9, 27). Arteether is prepared from artemisinin by etherification with ethanol in the presence of Lewis acid and separated from its chromatographically more slowly moving α-dihydroqinghaosu ethyl ether (3) and has been chosen by the Steering Committee of the Scientific Working Group on Malaria Chemotherapy of the World Health Organization for development because of better bioavailability than the parent drug. The compound is lipophilic and therefore has the potential to be effective against central nervous system infection, although this has not been studied. Congenital toxoplasmosis and toxoplasmosis in immunocompromised individuals often involve the central nervous system, and thus the lipophilic properties of arteether may be particularly beneficial in these settings. Cycloguanil is the active metabolite of proguanil. It is a dihydrofolate reductase inhibitor and is structurally similar to pyrimethamine (14). Cycloguanil was studied because, although there is only limited clinical experience with it, the parent drug, proguanil, has been used extensively and is considered the safest of the currently employed antimalarial agents (22, 24). Additionally, cycloguanil has been used to treat pregnant women with cutaneous leishmaniasis without adverse effects on the fetus (14). Primaquine phosphate was studied because of its antimalarial radical curative properties and minimal toxicity in persons who are not glucose-6-phosphate dehydrogenase deficient (27). Quinine sulfate remains highly effective against chloroquine-resistant malaria, including cerebral malaria, and has only minimal toxicity to bone marrow (27). Mefloquine is a recently licensed antimalarial agent with minimal apparent toxicity and an unknown mechanism of action (10, 27). Calcium and calmodulin antagonists have been shown to inhibit *Plasmodium falciparum* in vitro (21). We have shown previously that the calmodulin inhibitor cyclosporin A inhibits *T. gondii* in vitro (12). Verapamil was studied as the prototype calcium channel blocker, and trifluoperazine hydrochloride appears to be one of the more potent calmodulin inhibitors active against malaria (21). As the synergistic effect of pyrimethamine and sulfadiazine has been important in the treatment of toxoplasmosis, those antimicrobial agents found to be effective alone were also tested for synergism. The ranges of achievable levels in serum for the antimicrobial agents reported in the literature (16, 20, 26, 27) were those tested: arteether, 0.01 to 20 μg/ml; quinine sulfate, 5 to 15 μg/ml; mefloquine, 0.25 to 5.6 μg/ml; primaquine phosphate, 0.1 to 0.2 μg/ml; verapamil, 0.25 to 1

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μg/ml; trifluoperazine hydrochloride, 0.4 to 3 ng/ml; and cycloguanil, 12 to 69 ng/ml.

MATERIALS AND METHODS

Antimicrobial agents. The following antimicrobial agents (obtained from the indicated manufacturers) were utilized: pyrimethamine (Burroughs Wellcome Co., Research Triangle Park, N.C.), sodium sulfadiazine (City Chemical Co., New York, N.Y.), mefloquine (Hoffmann-La Roche Inc., Little Falls, N.J.), quinine sulfate (Warner Chilcott Laboratories, Morris Plains, N.J.), primaquine phosphate (Winthrop Pharmaceuticals, New York, N.Y.), trifluoperazine hydrochloride (SK&F Co., Philadelphia, Pa.), and verapamil (G.D. Searle & Co., Chicago, Ill.). Arteether and cycloguanil were obtained from the chemical inventory of the Walter Reed Army Institute of Research. All dilutions were made in medium 199 (M199; Grand Island Biological Company [GIBCO], Grand Island, N.Y.), with the exception of pyrimethamine and cycloguanil, which were initially dissolved in ethanol, and arteether, which was initially dissolved in dimethyl sulfoxide (DMSO; Sigma Chemicals, St. Louis, Mo.) and ethanol. Pyrimethamine was initially dissolved at a concentration of 10 mg/ml and cycloguanil was dissolved at 2 mg/ml. M199-fetal calf serum (M199-FCS) contained 10% heat-inactivated (60 min, 56°C) fetal calf serum (GIBCO), penicillin (100 U/ml), and streptomycin (100 μg/ml). M199-FCS containing the amounts of ethanol necessary to dissolve the pyrimethamine and cycloguanil was shown to have no effect on *T. gondii* in separate experiments. Arteether was initially dissolved in DMSO at a concentration of 5 mg/ml and then diluted to a concentration of 1 mg/ml in ethanol. M199-FCS containing the amount of DMSO and ethanol necessary to dissolve the arteether was shown to have no effect on *T. gondii* in separate experiments. Dilutions were prepared just prior to each experiment. The following concentrations of each agent were tested: arteether, 0.02, 0.2, 0.25, 0.5, 1.0, and 2.0 μg/ml; quinine sulfate, 2, 10, and 20 μg/ml; trifluoperazine hydrochloride, 0.5, 5, and 10 μg/ml; mefloquine, 0.01, 1.0, and 10 μg/ml; primaquine phosphate, 0.05, 0.5, and 5.0 μg/ml; cycloguanil, 1.0, 2.5, 5.0, 10, 25, and 100 μg/ml; and verapamil, 5, 10, and 50 μg/ml. In experiments to test synergy, concentrations of cycloguanil were 1, 2.5, 5, 10, 25, and 50 μg/ml alone and in combination, the concentrations of cycloguanil and sulfadiazine were 2.5 and 25 μg/ml, respectively.

Evaluation of effect of antimicrobial agents on macrophage and enterocyte cultures. Monolayers were visually inspected and graded in order to assess the effects of the various concentrations of antimicrobial agents on the cell cultures. Cell integrity was scaled from 1 to 4, with 4 being completely intact compared with that of the control and 1 being disrupted compared with that of the control. Additionally, cell cultures were incubated with [^{3}H]thymidine (Amersham), without an antimicrobial agent and with each concentration tested in order to determine the effects of the antimicrobial agent and with each concentration tested in order to determine the effects of the antimicrobial agents on the enterocytes' uptake of thymidine as an indication of the effects on the cell cultures.

Assessment of effect of antimicrobial agents on *T. gondii*. The effect of antimicrobial agents on *T. gondii* was evaluated as previously described (12). Briefly, an enterocyte cell line (13) or mouse peritoneal macrophages (12) were used for experiments. Peritoneal exudate cells from Swiss Webster female mice (Laboratory Supply, Indianapolis, Ind.) weighing 20 g each were cultured as previously described (12). After harvesting, peritoneal exudate cells were suspended at a concentration of 4 × 10^6 cells per ml in M199-FCS. Enterocytes were cultured at a concentration of 2 × 10^6 cells per ml. A 0.1-ml amount of the cell suspension was placed into each well of 96-well plates (0.28 cm^2; Linbro Scientific Inc., Hamden, Conn.). Cultures were incubated for 1 to 4 h at 37°C before challenge. Nonadherent cells were not removed from the cultures, because eliminating this step simplified performance of the assay and did not alter the results. M199-FCS (0.05 ml) that contained 4 × 10^6 tachyzoites of the RH strain of *T. gondii* was added to each well containing peritoneal exudate cells in 0.1 ml of M199-FCS. One hour after the addition of antimicrobial agents, 25 μl of M199 containing 2.5 μCi of [5,6-^{3}H]uracil was added to each well, and cultures were then incubated for 20 h. Cells were dislodged by alternating vigorous washing and aspirating with isotonic saline and collected on glass filters (9934-Ah; Whatman, Inc., Clifton, N.J.) with a multiple automated sample harvester (MASH II; M.A. Bioproducts, Walkerville, Md.). Filters were dried, and material retained by the filters was counted with a liquid scintillation spectrophotometer.

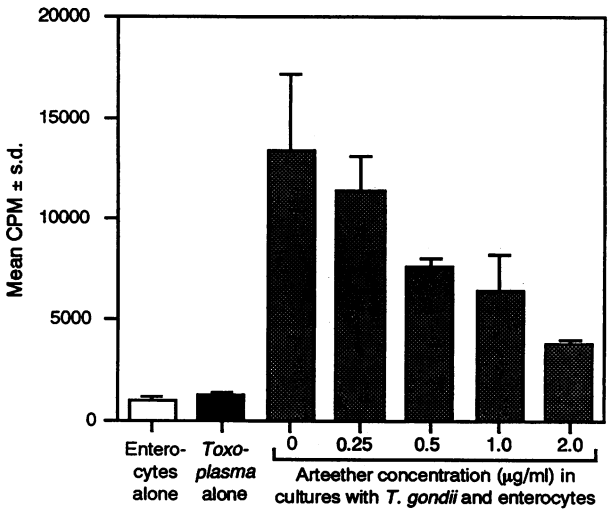

FIG. 1. Inhibitory effect of arteether on *T. gondii*. Data are means ± standard deviations (s.d.) of triplicate cultures from one representative experiment of three. Differences between untreated cultures and cultures treated with 0.5, 1.0, or 2.0 μg of arteether per ml were significant ($P < 0.05$).

Statistics. The significance of differences was evaluated by the multiple-range test of Neumann Keuls or Student's *t* test. *P* values of ≤0.05 were considered significant.

RESULTS

Arteether. Inhibition of *T. gondii* by arteether was indicated by a reduction in the incorporation of radiolabeled uracil into the nucleic acids of *T. gondii* tachyzoites. Arteether concentrations of ≥0.5 μg/ml were inhibitory to *T. gondii* ($P < 0.05$) (Fig. 1).

Cycloguanil. Cycloguanil concentrations of ≥1.0 μg/ml were inhibitory to *T. gondii* ($P < 0.05$) (Fig. 2).

Cycloguanil and sulfadiazine. The addition of noninhibitory concentrations of sulfadiazine (25 μg/ml) and cycloguanil (2.5 μg/ml) inhibited *T. gondii* more than this concentration of cycloguanil alone (Fig. 2).

Verapamil and trifluoperazine hydrochloride. These agents appeared to have no effect on *T. gondii* at the lower concentrations used (Table 1). At higher physiologic concentrations,

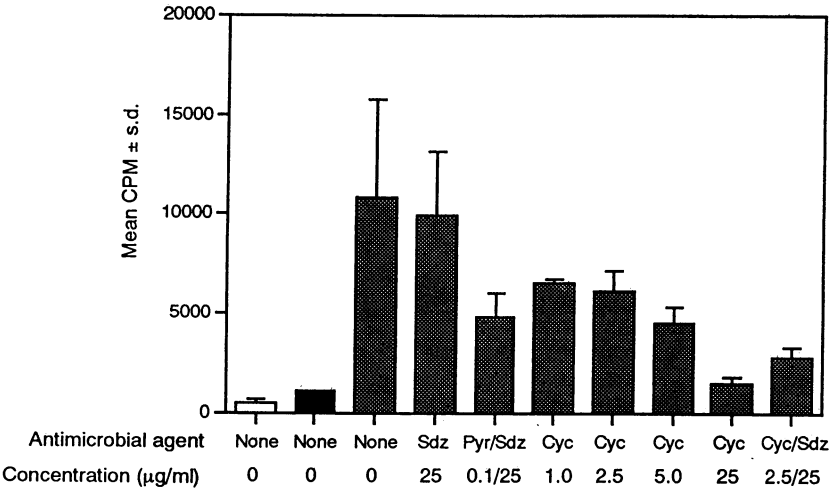

FIG. 2. Inhibitory effect of cycloguanil on *T. gondii* and synergistic inhibitory effect of cycloguanil and sulfadiazine. □, enterocytes alone; ■, *T. gondii* alone; ▩, enterocytes and *T. gondii*. Abbreviations: Sdz, sulfadiazine; Pyr, pyrimethamine; Cyc, cycloguanil. Data are means ± standard deviations (s.d.) from one representative experiment of seven. Differences between untreated cultures and cultures treated with ≥1.0 μg of cycloguanil per ml and with 2.5 μg of cycloguanil plus 25 μg of sulfadiazine per ml were significant ($P < 0.05$).

they appeared to reduce the uptake of uracil. It was not possible to assess their antimicrobial effects at these concentrations, since they were toxic to the cells in culture and destroyed the monolayers.

Primaquine phosphate, mefloquine, and quinine sulfate. Neither primaquine phosphate, mefloquine, nor quinine sulfate had any inhibitory effect on *T. gondii* ($P > 0.05$). Mefloquine had no effect at the usual achievable level in serum of 1 μg/ml (26, 27). It was toxic to the host cells at 10 μg/ml.

DISCUSSION

Effects of arteether, verapamil, trifluoperazine hydrochloride, mefloquine, cycloguanil, quinine sulfate, and primaquine

TABLE 1. Effects of antimicrobial agents on uracil uptake by intracellular *T. gondii* and on the host cells[a]

Antimicrobial agent	Concn (μg/ml)	Reduction in uracil uptake (%)	Monolayer density[b]	Thymidine uptake by unchallenged host cells (cpm ± SD)[c]
None	0		4	48,307 ± 4,164
Pyrimethamine + sulfadiazine	0.01, 0.25	52[d]	4	73,272 ± 7,502
Arteether	0.02	0	4	45,900 ± 3,026
	0.2	0	4	47,450 ± 4,219
	2	43[d]	3	54,879 ± 8,941
Quinine sulfate	20	0	4	42,854 ± 3,806
Trifluoperazine hydrochloride	0.5	0	4	46,303 ± 4,062
	5	45[d]	2–3[e]	36,543 ± 1,281[e]
	10	98	2[e]	38,800 ± 1,269[e]
Mefloquine	0.01	0	4	46,839 ± 3,926
	1	0	3	53,204 ± 1,899
	10	97	2[e]	36,102 ± 9,130[e]
Primaquine phosphate	0.05	0	4	47,153 ± 4,154
	0.5	0	4	50,185 ± 3,012
	5	0	4	46,172 ± 3,507
Cycloguanil	1	0	4	57,360 ± 1,868
	10	38[d]	3	53,472 ± 1,462
Verapamil	5	5	4	45,396 ± 2,180
	10	0	4	42,771 ± 4,395
	50	71	2[e]	25,346 ± 435[e]

[a] The uracil data and visual inspection of monolayers are from one representative experiment which was repeated two to four times with similar results. The thymidine data are from one experiment performed in conjunction with one of these experiments and confirmed in at least three experiments when significant effects of the antimicrobial agents on *T. gondii* replication were noted in the initial experiments.

[b] Monolayers were graded independently by two obscrvers using a scale between 1 (disrupted) and 4 (undisrupted control), and grading by the two observers was consistent.

[c] Thymidine uptake by cells exposed to the concentration of the antimicrobial agent (means ± standard deviations of triplicate cultures).

[d] Statistically significant diminution in uracil uptake compared with uptake by cultures without antimicrobial agents.

[e] Substantial disruption of monolayer which makes uracil uptake data uninterpretable.

phosphate on *T. gondii* within macrophages and/or enterocytes in vitro were studied by a micromethod we developed previously (12). Arteether is an ethyl ether derivative of qinghaosu and represents a unique class of antimalarial agents with a 1,2,4-trioxane ring. It was of interest to study arteether because qinghaosu is very effective in treating the related apicomplexan chloroquine-resistant malaria and especially cerebral malaria (3, 9–11). Administration of 2 mg of the arteminisinin derivative sodium artesunate per kg of body weight to patients with severe malaria resulted in plasma drug levels of 0.5 to 2.6 μg/ml (1, 27). Since artemisinin is actively incorporated into infected erythrocytes (8), artemisinin-like drugs demonstrate remarkable intrinsic antimalarial activities in vitro with 50% inhibitory concentrations ranging from 0.15 to 32 ng/ml (17, 28). In our studies, arteether concentrations of ≥0.5 μg/ml were inhibitory and this concentration was achievable in the studies of Benakis et al. (1). In animal models, toxicity has been observed (2) and the potential for neurotoxicity remains a serious concern for the development and use of arteether. It is important to note that arteether activity against *T. gondii* was significantly less than has been reported against falciparum malaria parasites in vitro; the 50% inhibitory concentration was 0.15 to 35.7 ng/ml (17, 28). In addition to exhibiting preferential uptake by infected erythrocytes, artemisinin is actively protein bound and lipophilic, and this may explain the marked differences between the activities in malaria-infected erythrocyte cultures and those in the cell culture monolayers reported here. If it is possible to determine the structure-activity relationships for neurotoxicity (25) with respect to the mechanisms of antiparasite action (15) of artemisinin derivatives or other synthetic peroxides, then it may be possible to design a better drug for toxoplasmosis as well as malaria. As we were performing this work, other investigators reported that arteether had no inhibitory effect on *T. gondii* in vitro (0.01 to 400 μg/ml) or in vivo (1 to 600 mg/kg of body weight) when administered subcutaneously to mice previously infected intraperitoneally with the RH strain of *T. gondii* (6, 7). However, as we were preparing the manuscript another group (18) reported results of the in vitro (0.4 to 4 μg/ml) effect of arteether on *T. gondii*, using a different in vitro assay system. This group's results (18) are similar to our findings. The reason(s) our results differ from those of Chang et al. (6, 7) is not clear. Since our results confirm those of Ou-Yang et al. (18), further testing of this antimicrobial agent and related congeners in in vivo models of toxoplasmosis is indicated.

The effect of cycloguanil on *T. gondii* was studied because it has been reported to be effective in prophylaxis against susceptible strains of malaria parasites as well as in treating cutaneous leishmaniasis (14, 27). Cycloguanil pamoate has been administered only parenterally, and its toxic effects since it was used only in an injectable form, included pain and tenderness at the site of injection, as well as reported allergy-like reactions with urticaria. These toxicities were sufficient to lead to discontinuation of its use for prophylaxis of malaria. However, proguanil is administered orally and cycloguanil is the active metabolite of proguanil. Thus, the oral formulation of proguanil now provides a less toxic way to deliver cycloguanil and makes it reasonable to determine whether it could be used as an alternative treatment for toxoplasmosis. In addition, although cycloguanil's structure is very similar to that of pyrimethamine and the mechanism of action is through inhibition of dihydrofolate reductase (4, 14, 27), one of the remarkable features of cycloguanil was that depression of hematopoiesis was reported only infrequently, and therefore it may prove to be less toxic than pyrimethamine (14, 22). Further, in areas where there is pyrimethamine-resistant malaria, proguanil is often still effective and proguanil has a different mechanism of action (4, 5, 14). Presumably, this is because mechanisms of resistance to pyrimethamine and cycloguanil are due to different point mutations in the dihydrofolate reductase molecule (19).

Despite cycloguanil use for over 40 years, studies of its pharmacokinetics have only recently been reported (22, 24). Cycloguanil has a relatively long half-life of 11 to 17 h, with peak concentrations 2 to 4 h after an oral dose of 200 mg of proguanil (24). Reported peak concentrations in serum and whole blood of cycloguanil following oral administration of proguanil were 12 to 69 ng/ml (24). Levels following intramuscular administration of cycloguanil have not been reported. The effect of cycloguanil alone on *T. gondii* and the synergism of the antimicrobial effects of cycloguanil with sulfadiazine that we demonstrated in vitro at these achievable concentrations in serum provide a basis for in vivo studies of its effect on toxoplasmosis.

Quinine sulfate and primaquine phosphate had no effect at the concentrations tested. The concentrations of verapamil and trifluoperazine hydrochloride tested were used because they approximated levels obtained in human serum when these drugs are used to treat other illnesses and/or had an inhibitory effect on malaria parasites at these concentrations (16, 20, 26, 27). The results of culture of *T. gondii*-infected cells with verapamil and trifluoperazine hydrochloride could be evaluated only at lower physiologic concentrations, since the higher physiologic concentrations destroyed the monolayers of host cells. Previous studies have documented the inhibitory effects of calmodulin inhibitors on related protozoa (21). Trifluoperazine hydrochloride and verapamil inhibit *P. falciparum* in vitro but have weak intrinsic activities relative to standard antimalarial agents. Chloroquine-resistant malaria became chloroquine responsive in vitro with the addition of verapamil, but the utility of such calcium antagonists in combined therapy (21) has been limited by poor pharmacokinetics and a potential for toxicity (23). Both calcium channel blockers and calmodulin inhibitors are thought to exert their effects through the inhibition of penetration of *P. falciparum* into the host cell. Mefloquine had no effect at the lower concentrations tested, which approximated those in previously reported pharmacokinetic studies of this antimicrobial agent (26, 27). The highest concentration (10 mg) tested was toxic to the host cells. Mefloquine's mechanism of action against malaria has not been reported.

Our study provides data which indicate that arteether and cycloguanil are effective in vitro against *T. gondii* when used as single agents and that cycloguanil and sulfadiazine are synergistic in vitro. The data reported herein provide a foundation for further studies to test the efficacy of these antimicrobial agents alone and together by using in vivo models and to determine whether they are also effective against encysted *T. gondii* bradyzoites. If they are effective in in vivo models, they may provide alternative therapies for toxoplasmosis.

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