Artemisinin, a peroxide-containing sesquiterpene lactone isolated from the herb Artemisia annua, has been found to possess potent antimalarial activity and low toxicity both in animals and humans.\(^1\)\(^2\) Artemethane (a water-soluble half-ester sucinate derivative) and artemether (a methyl ether derivative) are the only 2 derivatives of artemisinin that have been licensed in Thailand for treatment of Plasmodium falciparum malaria since 1990.

Because of their low solubility in either water or oil\(^3\) and the short plasma half-life of artemisinin, artemate, and artemether have been studied, in particular, sodium artemate.\(^4\) All artemisinin derivatives are metabolized to an active metabolite, dihydroartemisinin, whose half-life is 2 hr compared with the 45 min half-life of artemate.\(^5\) Artemesate and dihydroartemisinin are active against severe or complicated P. falciparum infection in humans and animals.

Artemisinin contains an endo-peroxide bridge. The peroxide moiety has been demonstrated to be responsible for the antimalarial activity of these compounds\(^6\)\(^7\) and presumably for antitoxoplasmal activity.\(^6\) Since these drugs cross the blood-brain barrier, they have been tested as a treatment for toxoplasmosis of the brain of an infected mouse. To our knowledge, these qinghao derivatives have been only tested on the RH strain, a highly virulent strain of Toxoplasma gondii.\(^8\)\(^9\)

The purpose of the present study was to evaluate the in vitro and in vivo effects of artemesate, dihydroartemisinin, and their combination on the cyst-forming strain of T. gondii.

**MATERIALS AND METHODS**

**Parasite.** Toxoplasma gondii strain DUR was isolated from the amniotic fluid of a pregnant woman. This isolate is considered to be low virulence because it causes a chronic infection in mice and grows slowly in culture. This avirulent strain was maintained in our laboratory by oral passage of cysts from the brain of an infected mouse.

**Cell culture.** The human myelomonocytic cell line THP-1 (European Collection of Animal Cell Cultures number 88081201; Sophia-Antipolis, France) was used for T. gondii culture. These non-adherent cells were suspended in RPMI 1640 medium (DAP, Vogelgrun, France) supplemented with 100 U/ml of penicillin, 100 \(\mu\)g/ml of streptomycin (Sigma-Aldrich, L’Isle d’Abeau, France), and 10% fetal calf serum (DAP). The number of THP-1 cells plus reference drugs. The addition of the drugs to final concentrations of 0.1, 0.5, and 2 \(\mu\)g/ml.

Sulfadiazine and pyrimethamine (Sigma) were used as positive controls. Control wells included medium alone and THP-1 cells plus reference drugs. The addition of the drugs in the medium was made after 3 days of cultivation. All drugs dilutions were made in sterile medium. Working dilutions were freshly prepared for each experiment in a constant final volume of 500 \(\mu\)l. After various incubation times at 37°C in a moist 5% CO\(_2\)-95% air atmosphere, the content of each well was aspirated, placed in Eppendorf (Hamburg, Germany) microtubes, and was centrifuged at 15,000 rpm for 2 min. (Avanti 30 centrifuge; Beckman, Gagny, France). The viable cells were counted by staining with ethidium bromide and acridine orange.\(^1\) The percentage of infected cells, as well as the number of extracellular parasites, was calculated after counting 1,000 cells from triplicate wells. The percentage of growth inhibition was calculated using the following formula: 100 – (\% in treated wells/\% in controls).

**In vivo studies.** Twenty female OF1 mice (8 weeks old) (IFAA-CREDO; L’Abresle, France) were infected by gavage with cysts obtained from the brain of an infected mouse. The brain tissue was suspended in 2 ml of 0.9% NaCl and ground with a mortar and pestle. The preparation was further homogenized by passage through a needle and syringe. The concentration of cysts was determined by bright-field light microscopy. The cysts were diluted with sterile saline solution to a final concentration of 10 cysts/0.2 ml/mouse.

**Drugs.** Artesunate and dihydroartemisinin were obtained from Dr. A. Benakis (Laboratory of Drug Metabolism, Department of Pharmacology, University Medical Center, Ge-
neva, Switzerland). Three months postinfection, the infected animals were divided into 2 groups of 10 each: untreated or control mice (group 1) and treated animals (group 2). The treated mice received an oral regimen of artemunate-dihydroartemisinin (50:50) 3 times a day at a dose of 100 mg/day in a 0.2-ml volume per animal for 5 days. The animals were killed 3 days after the end of treatment.

The brain of each mouse was removed for counting of cysts and microscopic and histologic studies. Half of the brain was used to evaluate the number of cysts in untreated and treated mice. The tissue was mixed with phosphate-buffered saline and ground as above. The number of cysts in six samples (20 μl each) was determined for each brain.

**Light microscopy.** Brain samples from untreated and treated mice were obtained randomly, incubated in Bouin’s fixative, dehydrated in a graded series of ethanol, and embedded in paraffin. Sections (4 μm) were stained with Giemsa or hematoxylin, phloxine, and safranin.

**Viability assays. In vitro.** After parasitized cells were incubated with different concentrations of the drugs for 48 hr at 37°C, the parasitized cells were washed 3 times with RPMI 1640 medium to remove both dead cells, parasites, and drugs. The cells were then incubated at 37°C for 72 hr.

**In vivo.** The viability of the cysts recovered from the 2 groups of mice was tested by gavage in 2 groups of 10 naive 8-week-old OF1 mice.

**Statistical analysis.** The significance of differences was evaluated using Student’s t-test. P values ≤ 0.05 were considered significant.

**RESULTS**

A preliminary in vitro study carried out with 0.1–2 μg/ml of artemunate and dihydroartemisinin alone or in combination showed a lack of toxicity to THP-1 cells. However, even at low concentrations, these drugs induced the death of the infected cells. At concentrations of 0.5 μg/ml, sulfadiazine and pyrimethamine resulted in about 80% inhibition of parasitized cells after 96 hr; while at the same concentration, artemunate resulted in approximately 40% inhibition, dihydroartemisinin resulted in 70% inhibition, and the combination resulted in approximately 65% inhibition (Table 1).

Dihydroartemisinin, as well as the artemunate-dihydroartemisinin combination, induced greater inhibition of parasite growth after 12 hr than after 96 hr. Maximum inhibition by artemunate was observed at 24 hr, and was always followed by a subsequent decrease in inhibition at 96 hr. Phase contrast microscopy showed that the number of tachyzoites after treatment with artemunate or dihydroartemisinin at a dose of 0.5 μg/ml was lower than that of the controls (35% versus 45%, respectively). After incubation for 48 hr with the artemunate-dihydroartemisinin combination, the number of tachyzoites was decreased about 55% compared with that of the controls. Treated tachyzoites appeared similar to those of the controls after treatment with either of the drugs or the drug combination, but they appeared to be in a latent, motionless state. However, they were apparently still viable because they appeared green after staining with ethidium bromide and acridine orange. Conversely, tachyzoites treated with spiramycin or pyrimethamine at the same dose were dying and thus stained orange or red. To show if the apparently motionless parasites were dead or alive, these cultures were washed after 48 hr of incubation with the various molecules. After 72 hr, approximately 10^7/ml of extracellular T. gondii and parasitized cells were observed.

No mice died during the in vivo experiments. The number (± SD) of T. gondii cysts found in the brains of mice treated with artemunate-dihydroartemisinin was lower than that found in the control brains (267 ± 10.31 and 650 ± 37.27, respectively). After 5 consecutive days of treatment, approximately 75% of the cysts did not appear different by phase contrast microscopy from those of the controls. However, their internal membranes seemed altered with an irregular outline, and approximately 25% of the bradyzoites were damaged.

Three months after transplantation, the number of cysts found in the cerebral tissues of mice gavaged with brain homogenates from treated animals was 40% lower than the number found in mice gavaged with brain homogenates from controls.

Histologic analysis showed a modification of the cerebral tissue due to the presence of the cysts. Compared with the controls, a disorganization of the tissue and a necrosis process were observed. Furthermore, there were modifications in the microscopic aspect of the cysts, which were smaller than the controls, and internal damage was observed (Figure 1).

**DISCUSSION**

The aim of this study was to investigate the effects of artemunate and its active metabolite dihydroartemisinin on cerebral toxoplasmosis. In our in vitro studies, the growth of an avirulent strain of T. gondii that was incubated for 96 hr with artemunate or its active metabolite was not completely inhibited. A maximum inhibition of 70% was observed after 96 hr of exposure to dihydroartemisinin. After a 48-hr exposure to the drugs and several washings of the cells, a rapid increase in parasite growth was observed. It appears that once the drugs are taken up, it is difficult to remove
them by washing the cells. This was also observed by Hassan Alin and Bjorkman. Artesunate and/or dihydroartemisinin triggered the death of the infected THP-1 cells, but the combination of the 2 drugs did not significantly improve the inhibition of parasite growth. Our hypothesis is that artesunate and dihydroartemisinin act in a similar way in comparison with their activity in P. falciparum-infected erythrocytes, but dihydroartemisinin is concentrated 300 times more than in the uninfected erythrocytes. Maximum uptake of artemisinin has been shown to occur after 1–3 hr. This resulted in the destruction of parasitized cells, but free parasites in the medium were minimally altered.

Chang and Perchère showed that in macrophage monolayers infected with the T. gondii RH strain and treated with arteether, an ether derivative of dihydroartemisinin, an inhibitory effect on Toxoplasma replication by decreasing the number of infected cells was observed; however, these results were not reproducible. Arteether concentrations >0.5 μg/ml were inhibitory 1 hr after the addition of the drug to the RH strain in in vitro assays with macrophage and enterocyte cultures. In vitro studies performed on fibroblasts infected with the RH strain showed a 100% reduction in parasite growth after treatment with arteether (1 μg/ml), a 100% reduction in parasite growth after treatment with arteether (0.1–1 μg/ml), and a 98% reduction in parasite growth after treatment with a lower concentration of arteether (0.01 μg/ml). We obtained similar results in this study with our cystic strain.

Despite these in vitro results, we chose to perform in vivo studies of a combination of artesunate and its active metabolite dihydroartemisinin to increase the plasma level of dihydroartemisinin, whose half-life is greater than that of artesunate, and thus, increase its cerebral concentration. Swiss-Webster female mice infected with the RH strain of T. gondii and treated subcutaneously with different daily doses of arteether showed more protection compared with mice treated orally with roxithromycin. These data were later confirmed by Brun-Pascaud and others, who showed that after 5 weeks of immunosuppression, rats inoculated with the RH strain of T. gondii and treated with arteether at doses of 18 and 100 mg/kg died within 8 days. These mice had been treated with a total dose of 500 mg over a 5-day period and treatment was well tolerated. Cooke and others showed that 1 hr after a 900 mg/kg oral dose was given, a concentration of 6 μg/g was observed in rat brain tissue. The total dose in our study was less than this value, but the number of cerebral cysts decreased by 59% in treated animals compared with the controls. Thus, this low cerebral dose displayed a certain efficacy.

Our microscopic study showed that cysts treated with the drugs generally appeared unchanged, but an alteration of their inner membranes occurred. Maeno and others demonstrated that the earliest pathologic effect of artemisinin is on the parasitic membrane in P. falciparum, which seems to be the case in our study. We used a short treatment duration because we wanted to know if the artesunate-dihydroartemisinin combination could act as rapidly against T. gondii as observed against P. falciparum. Given this short period, the drugs show an interesting efficacy; however, some brain cysts in the cerebral tissue were still viable because they were able to produce secondary cysts in naive mice. We obtained a better inhibition of parasite growth results in vivo with our cyst-forming strain than with the RH strain, but in vitro it was lower than that previously observed by other
in conclusion, our results show an increase in efficiency for the artesunate-dihydroartemisinin combination over a short period of time, but a possible recrudescence of parasite growth both in vitro and in vivo. This could limit the use of these drugs in the treatment of encephalitis due to *Toxoplasma* because there are other more active drugs (both in vitro and in vivo), such as 2',3'-dideoxyinosine.\(^2,20\)

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