

# IN VITRO EFFECT OF ARTESUNATE AGAINST ACANTHAMOEBA SPP

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**Abstract.** The *in vitro* effects of artesunate, the antimalarial agent, and metronidazole against *Acanthamoeba* spp were studied. *Acanthamoeba* Group II and *Acanthamoeba polyphaga*-like were isolated from natural water courses in Buri Ram Province, northeastern Thailand. The trophozoites were axenically cultured in PPYG medium and treated with artesunate in a concentration of 5-700 µg/ml. Artesunate showed its ability to inhibit the growth of acanthamoeba trophozoites: 54% at 50 mg/ml (after six days of exposure) and 93.2% at 100 µg/ml (after two days). The 500-700 µg/ml concentration caused inhibition on the first day of more than 93.2%; excystation did not occur in drug-treated medium. The present study shows that artesunate is amebastatic rather than amebicidal in an axenic culture of trophozoites at the highest concentration of 100 µg/ml. Metronidazole, in concentrations of 5-1,000 µg/ml, had no effects on either trophozoites or cysts.

## INTRODUCTION

*Acanthamoeba* is a genus of small free-living amebae that are ubiquitous in nature. The organisms are opportunistic pathogens that cause several rare diseases in humans, which include the disease of the central nervous system known as granulomatous amebic encephalitis (GAE), the sight-threatening eye disease known as *Acanthamoeba* keratitis (AK), and various secondary infections associated with immunocompromised individuals such as AIDS patients. *Acanthamoebae* have also been associated with disease in a variety of animals other than humans (CDC, 1986; Kilvington and White, 1990; Sison *et al*, 1995; Martinez and Visvesvara, 1997; Bonilla *et al*, 1999). Infection and colonization by *Acanthamoeba* in these hosts have been on the increase and, consequently, there has been renewed interest in finding drugs that effectively inhibit or eliminate these amebae from their human hosts. A number of agents have been used to treat these infections (Bradley, 1996; Chu *et al*, 1998; Schuster and Visvesvara, 1998). However, *Acanthamoeba* can form cysts in human tissue, which can be difficult to treat effectively because they are impervious to a number of drugs; furthermore, cysts can be transformed into active trophozoites following the cessation of therapy (Osato *et al*, 1991; Hay *et al*, 1994). In recent studies, the efficacy of artesunate has been shown in many other parasitic infections, such as cerebral malaria, *Toxoplasma gondii*, *Schistosoma mansoni*, *S. japonicum*, *Clonorchis sinensis*, *Opisthorchis viverrini*,

and *Gnathostoma spinigerum* (Chen *et al*, 1983; Klayman, 1985; Xio and Catto, 1989; Ke *et al*, 1991; Lee and Andrew, 1995). The aim of the present study was to evaluate the *in vitro* efficacy of artesunate in comparison with metronidazole against the trophic and cystic forms of *Acanthamoeba* spp.

## MATERIALS AND METHODS

### Amebic isolation and culture

Amebae were isolated from natural water courses in northeastern Thailand (Nacapunchai *et al*, 1999) and designated as having *Acanthamoeba polyphaga*-like morphology or being in group II as defined by Page (1988).

Trophozoites were axenically cultured with PPYG medium in 25 cm Corning flasks and incubated at 35°C (Schuster and Visvesvara, 1998). Trophozoites were collected from exponential growth (72 to 96 hours) and tested immediately.

Cysts were obtained from two-week subcultures of trophozoites on 1.5% non-nutrient agar plates at 35°C. The cysts were harvested and washed in phosphate-buffered saline.

Trophozoites or cysts in the resultant suspensions were concentrated by centrifugation at 350g for 10 minutes, and then counted in a hemacytometer and adjusted to a final concentration of 10<sup>4</sup> trophozoites or cysts per milliliter.

### Experimental design

The stock solutions of artesunate (ATC; Guilin No2 Pharmaceutical Factory, Guangxi, China) and metronidazole (Flagyl®; May & Baker Ltd, Dagenham, England) were prepared in final concentrations of 5 mg/ml and 10 mg/ml respectively. The solutions were then diluted in medium for use in the assays. The final concentrations of artesunate were 5, 10, 20, 50, 100,

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200, 300, 400, 500, 600 and 700 µg/ml. Those of metronidazole were 5, 50, 200, 400, 800 and 1,000 µg/ml. Each concentration was tested in triplicate.

For the trophozoite and cyst assays, 500 µl of calibrated amebic suspension was added to each well of a 24-well culture plate (Cel-Cult®; Sterilin Ltd, Felhem, England) and then 500 µl of drug or control medium was added to make the final concentrations described above. The plates were observed daily for two weeks by visual assay using light inverted microscopy (x20 and x40 objectives). After drug exposures, the amebae (both trophozoites and cysts) were washed and resuspended in fresh medium (minus drug) in order to test their viability.

**Statistical analysis**

Student's *t*-test was used when comparing results between two treatment groups.

**RESULTS**

**Effects of the drugs on trophozoite growth**

Fig 1 illustrates the growth inhibition of artesunate on vegetative forms: 54% at 50 µg/ml (days 6 to 14) and 93.2% at 100-700 µg/ml (from day 2). Encystment was induced at 50-700 µg/ml (days 6 to 14) which was not significantly different (*p*>0.05) in all the concentrations of the drug. The ameba culture, after

washing-out of the drug, returned to normal levels within seven days. Metronidazole had no effect on the trophozoite growth (Fig 2).

**Effects of the drugs on cystic forms**

Artesunate showed only cystostatic effect on *Acanthamoeba* spp at concentrations of 500-700 µg/ml. Metronidazole had no effect on the cysts.

**DISCUSSION**

Satisfactory treatments for AK and GAE are lacking. A variety of drugs has been used clinically, but no one agent has been shown to be effective against all *Acanthamoeba* isolates. Treatment of human infections with propamide isetionate and dibromopropamide has reportedly produced a cure for some patients, whereas other investigators have documented a progression of the disease in other patients (Auran *et al*, 1987; Binder, 1989). Recently, artesunate, an established antimalarial has been studied for its effect against *Toxoplasma gondii*, *Schistosoma mansoni*, *Clonorchis sinensis*, and *Opisthorchis viverrini* and was found to be partially effective (Chen *et al*, 1983; Xio and Catto, 1989; Ke *et al*, 1991; Laha, 1994). In the present study, artesunate showed amebastatic but not amebicidal activity which may due to the inhibition of *Acanthamoeba* DNA synthesis as

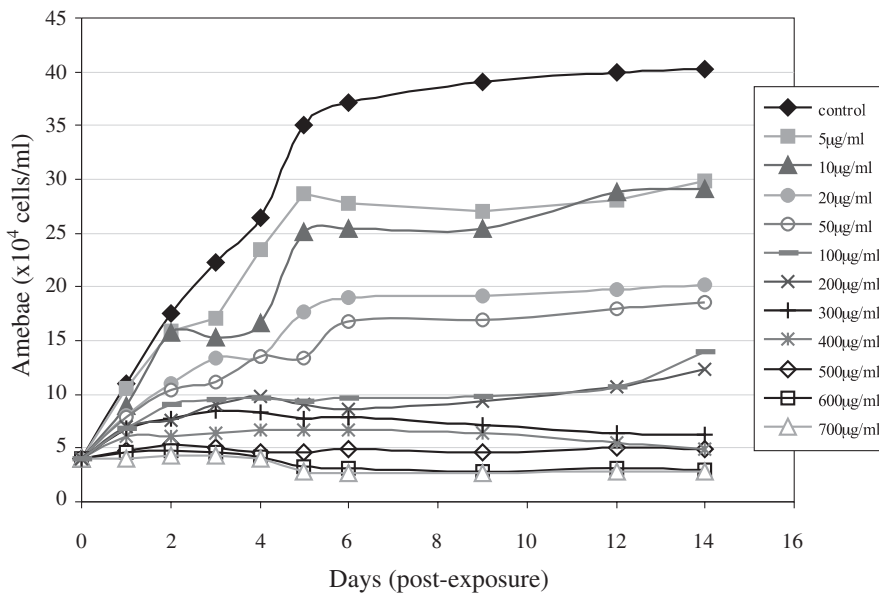


Fig 1- Growth of *Acanthamoeba* spp in the presence of artesunate 5-700 µg/ml. The control group represents the amebae grown in the absence of the drug.

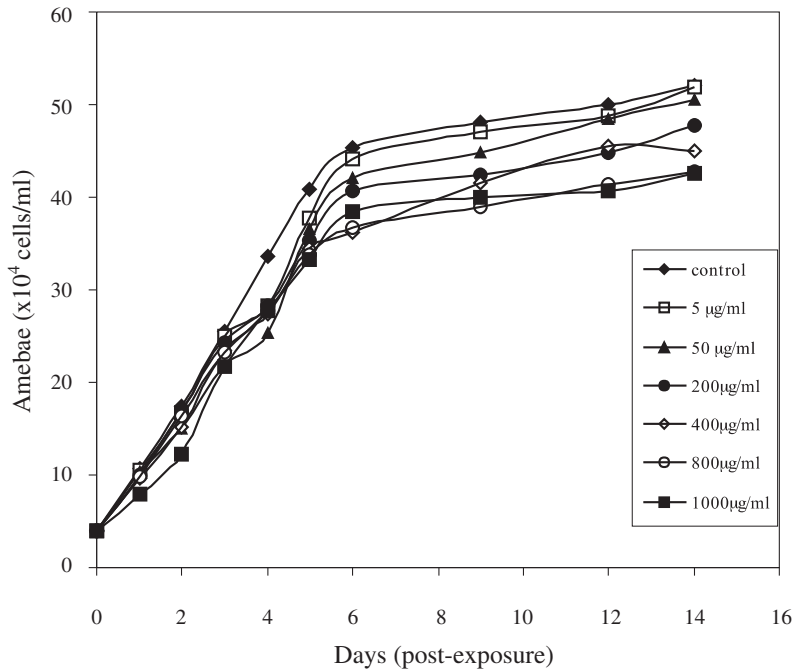


Fig 2- Growth of *Acanthamoeba* spp in the presence of metronidazole 5-1,000 µg/ml. The control group represents the amebae grown in the absence of the drug. The differences in all groups were not significant.

found in malaria and *Toxoplasma gondii* (Ke *et al*, 1991; Lee and Andrew, 1995). Encystment could be induced by artesunate in the culture even in low number of cysts, which shows some effect of the drug on cell differentiation and the metabolic inhibitors that induce encystment; furthermore excystation was not evident in the presence of the drug. In contrast, metronidazole had no activity against *Acanthamoeba*, as previously described (Freeman *et al*, 1997). Our data do not suggest any particular mechanism by means of which artesunate exerted its effects on the amebae. In early studies, encystment of *Acanthamoeba* developed in response to several antibiotics that affected mitochondrial processes (Seilhamer and Byers, 1978; Akins and Byers, 1980; Byers *et al*, 1981). Other investigators have noted that each *Acanthamoeba* isolate demonstrates different sensitivities to a specific agent (Tomlinson, 1991). Our result needs to be confirmed by studies of other amebic strains and species, in association with other drugs *in vitro*, and in experimental animal models.

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