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Artemether administered together with haemin damages schistosomes *in vitro*

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Abstract

We conducted experiments *in vitro* to assess the effect of artemether in combination with haemin on adult *Schistosoma japonicum*, *S. mansoni* and *S. haematobium*. When schistosomes were maintained in a medium containing artemether at concentrations of 20 μg/mL or less for 72 h, no apparent effect on the schistosomes was seen. When the medium contained 50 or 100 μg/mL haemin as well as artemether, the schistosomes showed decreased motor activity 2–24 h after exposure, which was followed by the staining of the whole worm body a reddish-yellow colour, dilatation of the intestine, and extensive vesiculation of the tegument. Some of the schistosomes died 24 h after exposure, and almost all died within 48–72 h. When schistosomes were exposed to the same concentrations of haemin alone, they were stained a light yellow colour but there was no apparent effect on their survival. Our findings suggest that artemether interacts with haemin to exert a toxic effect on the worms, which might be of importance in the further elucidation of the mechanism of action of artemether on schistosomes.

Keywords: schistosomiasis, *Schistosoma japonicum*, *Schistosoma mansoni*, *Schistosoma haematobium*, artemether, haemin

Introduction

Artemether, a derivative of artemisinin, is used effectively in the treatment of severe malaria and multidrug-resistant falciparum malaria (McINTOSH & OLLIARO, 1999). Summarizing the data dealing with the interaction of artemisinin with haemin, heme and free iron, a two-step mechanism of action against malaria parasites has been proposed (KAMCHONWONGPAISAN & MESHNICK, 1996; MESHNICK *et al.*, 1996; MESHNICK, 1998; DHINGRA *et al.*, 2000). Artemisinin is activated by intraparasitic iron, which catalyses the cleavage of the endoperoxide bridge and the generation of free radicals. This is followed by alkylation and the formation of covalent bonds between artemisinin-derived free radicals and parasite protein(s).

During the early 1980s, artemether was also found to be active against *Schistosoma japonicum* (see LE *et al.*, 1982). Detailed laboratory studies showed that the juvenile stages of the parasite were more susceptible than the adult worms (XIAO *et al.*, 1995, 1998a). Consequently, the use of artemether for prophylaxis against *S. japonicum* was developed through experimental studies and a series of clinical trials in humans (for a review, see XIAO *et al.*, 2000a). Laboratory studies also showed that artemether has a prophylactic effect against *S. mansoni* (see XIAO & CATTO, 1989; XIAO *et al.*, 2000b) and *S. haematobium* (see XIAO *et al.*, 2000c). Recently, the laboratory findings with *S. mansoni* have been confirmed in a randomized controlled trial in humans (UTZINGER *et al.*, 2000), and a similar trial is currently under way with *S. haematobium*.

In our earlier work, artemether was found to exert various effects on schistosomes *in vivo*. Movement of worms to the liver (hepatic shift) started 8 h after drug administration and reached a peak after 72 h (XIAO & CATTO, 1989). Artemether had extensive effects on carbohydrate, nucleic acid and protein metabolism of the schistosomes, and enzymes related to the corresponding biochemical process were markedly inhibited after 48–72 h (YOU *et al.*, 1994; XIAO *et al.*, 1997, 1998b, 1998c, 1999; ZHAI *et al.*, 1999). On the other hand, artemether at concentrations less than 100 μg/mL had no apparent action on schistosomes *in vitro* (XIAO & CATTO, 1989). It was therefore interesting to investigate the mechanism of the observed *in vivo* effects.

Since blood cells are taken up by schistosomes about 2 weeks after entry into the mammalian host (CLEGG, 1965), and pigment resulting from the metabolism of haemoglobin is formed in their intestines (KLOETZEL & LEWERT, 1966), it is possible that an interaction between artemether and iron-containing metabolites of haemoglobin may occur. By analogy with the assumed mechanism of action of artemisinin antimalarial compounds, the generation of free radicals (BUTLER *et al.*, 1998) could also play a role in killing the schistosomes. The present paper represents a first step to investigate *in vitro* the possible effect of artemether in combination with haemin on schistosomes, which may be of relevance in understanding the possible mechanism of its action.

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Materials and Methods

Parasites

S. japonicum cercariae (Anhui isolate), released from infected *Oncomelania hupensis* snails, were provided by the Institute of Parasitic Diseases, Chinese Academy of Preventive Medicine (Shanghai, China). *S. mansoni* cercariae (Liberia isolate), released from infected *Biomphalaria glabrata* snails, were provided by the Swiss Tropical Institute (Basel, Switzerland). *S. haematobium* cercariae (Côte d'Ivoire isolate) were obtained after the first experimental passage through the sympatric snail intermediate host *Bulinus truncatus*. The snails were kept and infected in the laboratory of UFR Bioscience, University of Cocody (Abidjan, Côte d'Ivoire) and then transferred to the laboratory of the Swiss Tropical Institute for release and collection of cercariae.

Host animals

Male and female Kunming strain mice, weighing 22–24 g, provided by Shanghai Animal Centre, Chinese Academy of Sciences (Shanghai, China), were used for infection with *S. japonicum*. Female MORO strain mice (specific pathogen-free), weighing 18–22 g, obtained from the Biotechnology and Animal Breeding Division of RCC Ltd (Füllinsdorf, Switzerland) were used for infection with *S. mansoni*. Four male hamsters, weighing 60–80 g, of the Lake LVG(SYR)BR strain, were also obtained from the Biotechnology and Animal Breeding Division of RCC Ltd and used for infection with *S. haematobium*. All animals were maintained on commercial rodent food.

Infection and collection of schistosomes

The Kunming strain mice were infected with 80–100 *S. japonicum* cercariae via the shaved abdominal skin. The MORO mice were infected subcutaneously with 80–200 *S. mansoni* cercariae. Hamsters were infected subcutaneously with 400 *S. haematobium* cercariae.

Mice infected with *S. japonicum* for 32–38 d and mice infected with *S. mansoni* for 42–56 d were killed by bleeding. The schistosomes were collected by perfusion (YOLLES *et al.*, 1947) with ice-cold Hanks's balanced salt solution (HBSS) from mesenteric veins and livers, rinsed

3 times with HBSS containing 300 iu/mL penicillin sodium and 300 iu/mL streptomycin and kept in an ice bath. Hamsters infected with *S. haematobium* were also killed by bleeding, 77 d after infection. The intact intestines of the hamsters were removed and placed in a Petri dish. Schistosomes were removed from the mesenteric veins under a stereoscopic microscope with small forceps, following a procedure described in detail elsewhere (XIAO *et al.*, 2000b).

Drugs and chemicals

Artemether was the product of Kunming Pharmaceutical Corporation (Kunming, China; lot no. 970801). A stock solution of artemether was prepared by dissolving 5 mg of the drug in 3 mL polyethylene glycol (PEG 400) and then adding 2 mL HBSS to give a stock solution with 1 mg artemether per millilitre. This was diluted with HBSS for use. Haemin was obtained from Fluka (Buchs, Switzerland). Fresh haemin solution was prepared by dissolving 5 mg of haemin in 1 mL 0·1 M NaOH and adding 3·95 mL of HBSS followed by 0·05 mL of 1 M HCl to adjust the pH to 7·2–7·4.

Schistosome incubation in vitro

The incubation methods used for *S. japonicum*, *S. mansoni* and *S. haematobium* were identical. HBSS supplemented with 10% heat-inactivated calf serum, 300 iu/mL penicillin sodium, 300 iu/mL streptomycin and 0·25 mg/L amphotericin B was used to maintain the schistosomes *in vitro*. 1·78–2·00 mL of the medium were added to each of the 24 wells of a Falcon plate, then 2 pairs of worms were placed in each well. The plate was incubated at 37°C in 95% air + 5% CO_2 for 20–30 min before addition of artemether at various concentrations (0·5, 2·5, 5, 10 and 20 μg/mL). After the schistosomes had been exposed to artemether for 2 h, 50 or 100 μg/mL haemin were added. The final volume in each well was 2·0 mL. Control wells contained worms and medium only, medium plus the same concentrations of either artemether or haemin, or 0·6–1·2% PEG 400 alone (depending on the artemether concentration: for 0·5–10 μg/mL artemether 0·6% PEG 400, for 20 μg/mL artemether 1·2% PEG); they were maintained for the same time as experimental worms in a final volume of 2·0 mL.

After addition of the above-mentioned substances, the plates were incubated continuously for 72 h. The different combinations of artemether plus haemin and concentrations of artemether or haemin alone were tested 2 or 3 times with adult worms of all 3 schistosome species.

Assessment of drug effects

After 2, 4, 6, 8, 18, 24, 48 and 72 h, motor activity, tegumental alterations and parasite survival were assessed by examination under an inverted microscope and recorded. Parasite death was defined as no motor activity during 2 min observation plus severe and extensive tegumental alteration (i.e., more than 50% of the tegumental surface showing severe and extensive alterations, including vesiculation and collapse of large vesicles).

Results

The results are summarized in Tables 1–3.

Effect of polyethylene glycol, haemin or artemether alone

After incubation in medium containing 0·6% or 1·2% polyethylene glycol or haemin at 50 or 100 μg/mL, all individuals of *S. japonicum*, *S. mansoni* and *S. haematobium* showed a normal appearance with no tegumental damage. A slight decrease in motor activity was seen in a few worms after 72 h exposure; however, all worms were still alive at the end of the experiments.

No apparent alteration either in motor activity or in the tegument was seen when the 3 different schistosome species were exposed to various concentrations of artemether alone, and all schistosomes survived 72 h of incubation.

Effect of artemether together with haemin

When adult *S. japonicum* worms which had been maintained in medium containing 0·5 μg/mL artemether for 2 h were exposed to haemin at 50 or 100 μg/mL, the motor activity of the worms decreased after about 24 h incubation and stiffness of the worm body was observed. Motor activity decreased about 6–8 h earlier if the worms were exposed to the same concentrations of haemin but higher concentrations of artemether (2·5, 5 or 10 μg/mL). When adult worms of *S. haematobium* or *S. mansoni* were exposed to 5 or 10 μg/mL artemether together with 50 or 100 μg/mL haemin, reduction in motor activity was observed earlier than with *S. japonicum*: after 2–4 h with adult *S. haematobium* and after 4–8 h with adult *S. mansoni* worms. Usually, decrease of motor activities was accompanied by a gradual decrease in intestinal movement.

Table 1. Mortality rates of adult *Schistosoma japonicum* after exposure *in vitro* to artemether or haemin, or both substances

	No. of worms initially[b]		No. of worms dead after incubation[b]					
			24 h		48 h		72 h	
Treatment[a]	m	f	m	f	m	f	m	f
Control (medium only)	6	6	0	0	0	0	0	0
Polyethylene glycol (0·6%)	6	6	0	0	0	0	0	0
Haemin (50)	6	6	0	0	0	0	0	0
Haemin (100)	6	6	0	0	0	0	0	0
Artemether (0·5)	4	4	0	0	0	0	0	0
Artemether (2·5)	4	4	0	0	0	0	0	0
Artemether (5)	6	6	0	0	0	0	0	0
Artemether (0·5) + haemin (50)	4	4	0	0	0	0	2	1
Artemether (0·5) + haemin (100)	4	4	0	0	1	0	4	4
Artemether (2·5) + haemin (50)	4	4	0	0	4	4	–	–
Artemether (2·5) + haemin (100)	4	4	0	0	4	4	–	–
Artemether (5) + haemin (50)	6	6	0	0	4	4	6	6
Artemether (5) + haemin (100)	6	6	2	2	6	6	–	–
Artemether (10) + haemin (50)	4	4	0	0	4	0	–	4

[a]Concentrations of artemether and haemin in μg/mL are given in parentheses.
[b]m = Male, f = female.

Table 2. Mortality rates of adult *Schistosoma mansoni* after exposure *in vitro* to artemether or haemin, or both substances

	No. of worms initially[b]		No. of worms dead after incubation[b]					
			24 h		48 h		72 h	
Treatment[a]	m	f	m	f	m	f	m	f
Control (medium only)	12	10	0	0	0	0	0	0
Polyethylene glycol (0·6%)	8	7	0	0	0	0	0	0
Haemin (50)	10	10	0	0	0	0	0	0
Haemin (100)	10	10	0	0	0	0	0	0
Artemether (5)	5	5	0	0	0	0	0	0
Artemether (10)	7	6	0	0	0	0	0	0
Artemether (5) + haemin (50)	5	3	0	0	1	1	3	2
Artemether (5) + haemin (100)	5	4	0	0	2	2	5	4
Artemether (10) + haemin (50)	8	6	0	0	5	4	8	6
Artemether (10) + haemin (100)	7	5	0	2	4	3	7	5

[a]Concentrations of artemether and haemin in μg/mL are given in parentheses.
[b]m = Male, f = female.

Table 3. Mortality rates of adult *Schistosoma haematobium* after exposure *in vitro* to artemether or haemin, or both substances

	No. of worms initially[b]		No. of worms dead after incubation[b]					
			24 h		48 h		72 h	
Treatment[a]	m	f	m	f	m	f	m	f
Control (medium only)	6	4	0	0	0	0	0	0
Polyethylene glycol (1·2%)	6	4	0	0	0	0	0	0
Haemin (50)	3	2	0	0	0	0	0	0
Haemin (100)	3	2	0	0	0	0	0	0
Artemether (10)	3	2	0	0	0	0	0	0
Artemether (20)	3	2	0	0	0	0	0	0
Artemether (10) + haemin (50)	3	2	1	0	3	2	–	–
Artemether (10) + haemin (100)	3	2	0	0	3	2	–	–
Artemether (20) + haemin (50)	3	2	3	2	–		–	–
Artemether (20) + haemin (100)	3	2	3	2	–	–	–	–

[a]Concentrations of artemether and haemin in μg/mL are given in parentheses.
[b]m = Male, f = female.

With *S. japonicum* and *S. mansoni* worms a further decrease was seen in motor activity after 24–48 h of incubation and vesicles appeared focally on the tegumental surface. The vesicles were initially small, but became enlarged and eventually collapsed, making the tegumental surface appear rough. Vesiculation reached a peak after 48–72 h. Similar observations were made with *S. haematobium*, but these events occurred approximately 24 h earlier. After 24–48 h incubation (with *S. japonicum* and *S. mansoni*) and 18–24 h (with *S. haematobium*), dilatation of the intestine was usually seen and both male and female schistosomes were stained a reddish yellow-brown colour, which made the worm body cloudy and indistinct. No such staining of the worms was seen in the corresponding control groups.

When adult *S. japonicum* or *S. mansoni* were exposed for 24 h to 10 μg/mL artemether together with 50 μg/mL haemin, all male and female worms survived. Exposure of *S. haematobium* for 24 h to the same concentrations of artemether and haemin resulted in the killing of one male worm. All surviving schistosomes showed very feeble movements of the oral sucker. After 48 h incubation at the same concentrations, all *S. haematobium* and most *S. mansoni* worms had died. Meanwhile, all males of *S. japonicum* had died, whereas all females survived these concentrations. Twenty-four hours later, the remaining *S. mansoni* and *S. japonicum* had also died.

Use of a higher concentration of artemether (20 μg/mL) and the same or twice the concentration of haemin resulted in rapid killing of adult *S. haematobium* within 24 h (Table 3). At any given artemether concentration, more rapid killing of schistosomes was observed at the higher haemin concentration. Finally, exposure of adult *S. japonicum* to an artemether concentration as low as 0·5 μg/mL and the higher concentration of haemin (100 μg/mL) still resulted in effective killing after 72 h (Table 1).

Discussion

It is unusual to find a chemical compound which has both antimalarial and antischistosomal properties. Derivatives of artemisinin and the immunosuppressant drug cyclosporin A are such compounds, and understanding their mechanism of action is of great importance (CIOLI, 1998). Significant progress has been made with regard to artemether, a methyl ether derivative of artemisinin (MESHNICK *et al.*, 1996; XIAO *et al.*, 2000a), whereas information on the mechanism of antiparasite action of cyclosporin A is still limited (BELL *et al.*, 1996). With regard to artemisinin and malaria, it is believed that 2 sequential steps are involved. Activation of artemisinin inside the malarial parasite by heme or other iron systems results in cleavage of endoperoxide bridges and generation of free radicals, and formation of covalent complexes

between these free radicals and malaria-specific proteins or specific receptors (MESHNICK *et al.*, 1991, 1993, 1996; ZHANG *et al.*, 1992; KAMCHONWONGPAISAN & MESHNICK, 1996; MESHNICK, 1998; DHINGRA *et al.*, 2000). Both the parent artemisinin compound and its more active derivative artemether (HIEN & WHITE, 1993) are rapidly metabolized to the biologically active dihydroartemisinin (WHITE, 1996; VROMAN *et al.*, 1999).

The mechanism of action of artemether on schistosomes is still largely unknown (XIAO *et al.*, 2000a). Our tests *in vitro* demonstrated that, when schistosomes were exposed to artemether at concentrations of 20 μg/mL or less together with 50 or 100 μg/mL haemin, there was a gradual decrease in worm motor activity, followed by severe tegumental damage, cloudiness of the worm body due to its being stained a deep reddish yellow-brown colour, dilatation of the intestine and, finally, death. Very similar observations were made with all 3 schistosome species studied, but the effect occurred more rapidly with adult *S. haematobium* worms than with adults of *S. mansoni* or *S. japonicum*. Since worms exposed to artemether or haemin alone at the same concentrations exhibited no marked change during the incubation, it seems probable that the interaction of artemether and haemin leads to worm damage.

Schistosomes, like malaria parasites, must ingest host haemoglobin for their nutrition. Schistosomes even contain cathepsins which are similar to malarial cathepsins and which function in the breakdown of host haemoglobin, resulting in pigment formation in the intestines of the worms (KLOETZEL & LEWERT, 1966). Therefore, it is reasonable to suggest that artemether could also be activated within schistosomes by heme or other iron compounds as it is in malaria parasites, producing toxic compounds and/or free iron radicals, as recently suggested by BUTLER *et al.* (1998).

Previous studies *in vitro* showed no effect on schistosomes after exposure to artemether at concentrations as high as 100 μg/mL (XIAO & CATTO, 1989). It is possible that this was due to the small amount of pigment within the schistosomes' intestines. In a previous study (KLOETZEL & LEWERT, 1966), the pigment content of one pair of adult *S. mansoni* was estimated to be equivalent to 0·5 μg of haemin, which was far less than the amount of haemin added to the medium in our present studies *in vitro*. On the other hand, schistosomes constantly ingest red blood cells *in vivo*, and these are relatively rapidly metabolized within the worm intestines with a turn-over rate of once every 3–4 h (LAWRENCE, 1973). Therefore, artemether could interact with the constant pigment formation in the schistosomes' intestines. Female schistosomes produce about 4 times more pigment (KLOETZEL & LEWERT, 1966), ingest significantly more haemoglobin (ZUSSMAN *et al.*, 1970), and take up red blood cells at a 10-fold higher rate (LAWRENCE, 1973) than male worms, which may explain previous observations that adult females are more susceptible to artemether than male worms (XIAO *et al.*, 2000b and in press).

We conclude that further research is needed to elucidate fully the mechanism of action of artemether on schistosomes, but the present findings, that artemether together with haemin has a harmful effect on schistosomes *in vitro*, is undoubtedly of relevance, as the use of artemether and other artemisinin derivatives offers new dimensions and potential for schistosomiasis control.

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Announcements

Low Priced Publications from Tropical Health Technology

Students and medical workers from developing countries can purchase medical books and laboratory learning aids from Tropical Health Technology at reduced prices, including:
Manson's Tropical Diseases, 20th edition
Color Atlas of Tropical Medicine and Parasitology, 4th edition
Lecture Notes on Tropical Medicine, 4th edition.

District Laboratory Practice in Tropical Countries, part 1 (454 pp.) by Monica Cheesbrough is an up-to-date practical bench manual, illustrated in colour and black and white. It includes a parasitology section and also chapters on the staffing, quality management and health and safety of district laboratories, equipment and ordering, clinical chemistry investigations, and the preparation of a training curriculum for district laboratory personnel.

Also available are other up-to-date textbooks covering tropical paediatrics, epidemiology, cardiology, nursing, midwifery, laboratory sciences, and solar energy. Training materials include laminated colour photomicrographs with text covering the microscopical diagnosis of tropical disease.

A publications list with prices (inclusive of postage) and order form can be obtained from Tropical Health Technology, 14 Bevills Close, Doddington, March PE15 0TT, UK; phone +44 (0) 1354 740 825, fax +44 (0)1354 740 013.

African Index Medicus (AIM) Programme
An International Index to African Health Literature and Information Sources

In order to give access to information published in or related to Africa and to encourage local publishing, the Association for Health Information and Libraries in Africa (AHILA), with the technical support of the World Health Organization, initiated a programme to create an international index to health literature generated in African countries: the *African Index Medicus*.

The creation of the regional index is a collaborative and participatory process. Firstly, African countries create national health databases using a common methodology. Local information services and products are provided for national health professionals. National production should ensure self-sufficiency and sustainability at country level and the tailoring of services according to local needs.

The various national databases are then merged into a regional database to which are added bibliographic records relating to health in Africa from other international existing sources such as WHO's WHOLIS, MEDLINE, POPLINE etc. to produce the *African Index Medicus* in printed or electronic form, eventually CD-ROM. It is distributed to African countries as part of an affiliated membership to AHILA for institutions outside the region.

At this stage, AHILA, with support from WHO, is looking for further sponsoring partners at bilateral level with African countries not yet participating in the Project. Sponsorship comprises equipment and training of stafff and could be part of an information component of a health related project in the country, which may also include use of communications and CD-ROM.

Further information can be obtained from Mrs Lucilda Hunter, The Library and Documentation Centre, WHO Regional Office for Africa, P.O. Box BE 773, Belvedere, Harare, Zimbabwe.