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**Original article**

# *In vitro* effect of current antimalarial drugs on the survival of paired *Schistosoma mansoni* adult worms and their egg production.

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**Abstract:** Some field trials have already demonstrated the high antischistosomal potential of combination therapies using Artesunate (ART) and current antimalarial drugs (Boulanger *et al.,* 2007; Mohamed *et al.,* 2009; Sissoko *et al.,* 2009). The antischistosomal effects of these drugs are noteworthy, especially when they are used for the treatment of malaria in schistosomiasis endemic areas. However, the antischistosomal effects of Amodiaquine (AQ), Primaquine (PQ), Chloroquine (CQ) and Pyrimethamine (Py) have never been assessed by *in vitro* incubation. The objective of the present study is to assess the *in vitro* effects of current antimalarial drugs on the egg productivity of adult worm pairs of *S. mansoni* and their survival times. The effect of the current antimalarial drugs Mefloquine (MQ), quinine (QN), AQ, PQ, CQ, Sulfadiazine (Sf) and Py on the egg output of adult worm pairs of *Schistosoma mansoni* and their survival times during *in vitro* culture were assessed at a concentration of 10 μg/ml. AQ, PQ, CQ and Py significantly inhibited the daily egg output of paired female worms at a concentration of 10 μg/ml during the 1 or 2-day *in vitro* cultivation. However, QN and Sf did not significantly affect the daily egg output during the 8-day incubation. One-day exposure to MQ killed all paired male and female adult worms. AQ and PQ significantly decreased the survival of both paired male and female worms during the 14-day incubation, but QN, CQ, Py and Sf did not exert any similar effect. The present result is consistent with an assessment of the antischistosomal effects of artemisinin-based combination therapy in malaria and schistosomiasis co-endemic areas.
**Keywords:** antischistosomal drugs; antimalarial drugs; Schistosoma mansoni; mefloquine; quinine; amodiaquine; primaquine; chloroquine; pyrimethamine; sulfadiazine.

## INTRODUCTION

Schistosomiasis is one of the major parasitic diseases in tropical and subtropical areas. In spite of sustained control efforts, an estimated 800 million people are still at risk and approximately 200 million people are currently infected with the disease [1]. Praziquantel (PZQ) has been widely used as a first-line drug for the treatment of schistosomiasis [2]. However, the extensive reliance solely on PZQ for schistosomiasis control raises concerns about the development of a tolerant and/or resistant parasite. Indeed, there is clinical evidence for the presence of PZQ-resistant schistosomes in Senegal and Egypt [3, 4]. Thus, the research and development of new antischistosomal drugs are urgently needed.

Artemisinin (ARS) derivatives such as artemether and artesunate (ART) are well known as antimalarial drugs and also show effectiveness for the treatment of schistosomiasis in combination with PZQ [5, 6]. The antischistosomal activity of ARS derivatives is high in the juvenile migratory stages of the parasite but low in the adult worm [5, 7, 8]. Recently, new compounds such as 1, 2, 4-trioxolanes [9], csysteine protease inhibitors [10] and oxadiazoles [11] have been introduced as promising antischistosomal compounds. So far, clinical trials have not been conducted to evaluate the therapeutic effect of these compounds on schistosomiasis.

ARS derivatives alone exert a low antischistosomal effect on adult worms. However, some field studies demonstrated the high antischistosomal effect of Artemisinin-based combination therapy (ACT) [12-14]. A few studies have been done on the antischistosomal effect of current antimalarial drugs. Keiser *et al.* reported the interesting finding that MQ [15], one of the current antimalarial drugs, re-

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duced the worm burden in mice infected with *Schistosoma mansoni* or *japonicum.* Furthermore, the *in vitro* effects of MQ against juvenile and adult *S. japonicum* were assessed by Xiao *et al.* [16]. However, the antischistosomal activity of other current antimalarial drugs has never been assessed by *in vitro* incubation. Since schistosomiasis is often geographically co-endemic with malaria [17], the ACT used to treat malaria may also affect the schistosomes harboured by malaria patients. Thus, the assessment of the *in vitro* antischistosomal effect of current antimalarial drugs is essential to determine the impact of ACT on schistosomiasis.

The objective of the present study is to assess the effect of current antimalarial drugs on the survival of adult worm pairs of *S. mansoni* and on their daily egg output during *in vitro* incubation.

## MATERIALS AND METHODS

*Chemicals and medium*

MQ•HCl, PQ•$2H_3PO_4$, Sf•Na and Py were purchased from Sigma (St. Louis, Missouri, USA). AQ • HCl was purchased from MP Biomedicals Inc. (Fountain Parkway Solon, Ohio, USA). CQ•$2H_3PO_4$ and QN • HCl • $2H_2O$ were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MQ and Py were diluted in ethanol to concentrations of 10 and 5 mg/ml, respectively, as stock solutions. The other antimalarial drugs were diluted in deionized water to a concentration of 10 mg/ml as stock solutions. Each drug was then added to NCTC 135 medium (Sigma, St. Louis, Missouri, USA) containing a 1% solution of antibiotics (Penicillin 5,000 units and Streptomycin 5,000 mg/l, Gibco, Langley, Oklahama, USA) at a concentration of 10 μg/ml (free base).

*Parasite strain*

A *Puerto Rican* strain of *S. mansoni* (NIH-Sm-PR-1 strain) was routinely maintained by passage through GN hamsters and *Biomphalaria glabrata* (Newton's NIH Puerto Rican/Brazilian M-line) snails in the Animal Research Center at the Institute of Tropical Medicine, Nagasaki University. At eight weeks post-infection, adult worms were obtained by the perfusion technique as previously described by Smithers and Terry [18], and washed twice with NCTC 135 medium.

*Incubation*

The incubation was conducted as previously described by Mitsui *et al.* [8], except for the difference in test drugs and incubation period. Briefly, 48 adult worm pairs of *S. mansoni* were randomly assigned to eight groups: control, MQ, AQ, PQ, CQ, QN, Sf and Py groups. Each paired adult worm was preincubated for one day in a single well of a 24-well multi-well plate (Sumitomo Bakelite Co. Ltd., Osaka, Japan) with 0.5 ml of NCTC 135 medium alone in 5% $CO_2$ incubator at a temperature of 37 ℃.

Each worm pair was subsequently transferred into a well containing 0.5 ml of NCTC 135 medium alone for controls or supplemented with each drug at a concentration of 10 μg/ml as a free base. The plates were continuously incubated in 5% $CO_2$ incubator at 37 ℃, and the media were exchanged once a day for a period of 14 days. The number of eggs produced daily by each paired adult worm was counted, and worm viability was also observed visually under a Nikon SMZ 800 stereoscopic microscope. The "dead or alive" status of adult worms was determined by the movement response of each worm upon stimulation with a needle, that is, worms that failed to respond to needle stimulation were classified as dead.

*Data analysis*

Data was analysed using Epi-Info software (Centers for Disease Control and Prevention, Atlanta, Georgia, USA). The median survival time of *S. mansoni* adult worms was calculated, and then the medians were compared using the log-rank test. The daily egg output per female adult worm was expressed as the arithmetic mean (±S.E.M). Comparisons of daily egg outputs between groups were performed using the Kruskal-Wallis test or Mann-Whitney U test.

## RESULTS

*The effect of antimalarial drugs on egg production of Schistosoma mansoni adult worm pairs*

The effect of antimalarial drugs on the daily egg output of adult worm pairs at a concentration of 10 μg/ml is shown in Table 1. The mean daily egg output during the one-day pre-incubation period in the control group was 54.0±34.2, which was not significantly different from that in the other 7 antimalarial drug groups (Kruskal-Wallis test, $P = 0.743$ for all comparisons). On the first day of incubation, the mean daily egg output reached a peak of 132.8±38.7 in the control group and gradually decreased daily thereafter. On the first day after exposure to drugs, the mean daily egg output was almost zero in the MQ group, and 4.5±1.8 in the AQ group, and 6.0±3.1 in the Py group. The mean daily egg output in the AQ and Py groups was significantly lower than that in the control group ($P < 0.01$ and $P < 0.05$). On the second day, the mean daily egg output was 119.3±30.5 in the control group. Meanwhile, the mean daily egg output in the PQ and CQ groups was 3.5±3.5 and 12.8±7.1, significantly lower than that in the corresponding control group ($P < 0.01$ and $P < 0.01$, respectively).

Table 1. Effects of current antimalarial drugs on the daily egg output of adult worm pairs of *Schistosoma mansoni* over a period of eight days. Six worm pairs were incubated *in vitro* with no drug for control and with Mefloquine, Quinine, Amodiaquine, Primaquine, Chloroquine, Pyrimethamine or Sulfadiazine at a concentration of 10 μg/ml (free base).

| Drugs | Mean daily egg output of six paired adult worms (±S.E.M.) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 day pre-incubation | 1 day | 2 days | 3 days | 4 days | 5 days | 6 days | 7 days | 8 days |
| Control | 54.0 (34.2) | 132.8 (38.7) | 119.3 (30.5) | 92.0 (13.7) | 44.0 (10.7) | 29.8 (7.1) | 11.8 (5.0) | 0.7 (0.5) | 0.0 |
| Mefloquine | 54.7 (16.1) | 0.8 (0.8)* | | | | | | | |
| Quinine | 53.8 (27.5) | 85.8 (37.6) | 119.5 (33.0) | 79.8 (24.6) | 23.8 (7.9) | 23.7 (5.9) | 11.8 (6.0) | 1.5 (1.3) | 0.0 |
| Amodiaquine | 47.3 (26.9) | 4.5 (1.8)* | 0.0 | | | | | | |
| Primaquine | 26.3 (13.1) | 16.3 (8.6) | 3.5 (3.5)* | 0.0 | | | | | |
| Chloroquine | 77.2 (25.2) | 46.2 (14.2) | 12.8 (7.1)* | 1.5 (1.0)* | 0.0 | | | | |
| Pyrimethamine | 58.0 (25.8) | 6.0 (3.1)** | 0.0 | | | | | | |
| Sulfadiazine | 107.7 (49.6) | 122.5 (24.2) | 103.7 (39.5) | 77.3 (27.6) | 46.2 (18.4) | 35.5 (14.4) | 19.2 (4.6) | 3.5 (2.0) | 0.0 |

Mann-Whitney U test was used for the statistical analysis. * $P < 0.01$, ** $P < 0.05$, compared with the corresponding control group.

Table 2. *In vitro* effect of antimalarial drugs on the survival time of adult worm pairs of *Schistosoma mansoni* at a concentration of 10 μg/ml (free base) over a period of 14 days.

| Treatment | Number of worm pairs | Median survival days (range) | | *P*-value |
|---|---|---|---|---|
| | | Male | Female | |
| Control | 6 | ND | ND | - |
| Mefloquine | 6 | 1* | 1* | - |
| Quinine | 6 | ND | ND | - |
| Amodiaquine | 6 | 6 (6-9)* | 5 (5-6)* | <0.05 |
| Primaquine | 6 | 12.5 (10-14)* | 6 (5-7)* | <0.01 |
| Chloroquine | 6 | ND | ND | - |
| Pyrimethamine | 6 | ND | ND | - |
| Sulfadiazine | 6 | ND | ND | - |

A long-rank test was used for the statistical analysis. **P*-value < 0.01, compared with the corresponding control group. ND, No death was observed in any of the worms over the period of 14 days.

Throughout the eight-day period of exposure to drugs, the mean daily egg output in the QN and Sf groups was not significantly different from that in the corresponding control group.

*The effect of current antimalarial drugs on the survival time of Schistosoma mansoni adult worm pairs*

The survival of adult worm pairs of *S. mansoni* was observed during *in vitro* incubation with antimalarial drugs at a concentration of 10 μg/ml (Table 2). Throughout the experimental duration of 14 days, all adult male and female worms remained alive in the control group without morphological alterations. Meanwhile, death was observed in all adult male and female worms exposed to MQ at a concentration of 10 μg/ml for one day. A large amount of debris was observed on the tegument surface of the dead male and female worms.

When male and female worms were exposed to AQ at a concentration of 10 μg/ml for one day, all worm pairs separated. Four out of six female worms were elongated and exhibited focal accumulation of gut pigment sparsely on their bodies. In addition, remarkable blebs were observed on the tegument surfaces of two female worms. These blebs disappeared during the two-day incubation. Up to seven days after exposure to the drug, all female worms were elongated to the point of death. During the two-day incubation, no morphological alteration was observed in any of the male worms. Thereafter, remarkable blebs were found in two out of six male worms, but these blebs disappeared within five days. By the fifth day, all male worms were swollen and a large amount of debris was observed on their teguments. Subsequently, the male worms gradually died with a median survival time of six days. Similar morphological alterations caused by AQ were observed in male and female worms treated with PQ, although the occurrence of alterations was more delayed than in the worms treated with AQ. Compared with the control group, AQ and PQ significantly reduced the survival time of male (median: 6 days, $P < 0.01$; 12.5 days, $P < 0.01$) and female worms (median: 5 days, $P < 0.01$; 6 days, $P < 0.01$). On the other hand, CQ did not affect the survival time of either male or female worms throughout the 14-day incubation, and no apparent morphological alterations were observed in either male or female worms. Furthermore, as shown in Table 2, both AQ and PQ significantly reduced the survival time of female worms as compared with that of male worms ($P < 0.05$, $P < 0.01$).

Throughout the 14-day incubation, all male and female worms were alive in the Sf, QN and Py groups. No morphological alterations of male or female worms were ob-

served in the Sf and QN groups. Up to 11 days after exposure to Py, no morphological alterations were observed in any of the male or female worms. Subsequently, slight morphological damage was observed on the tegument of the male worms.

## DISCUSSION

Xiao *et al.* showed that when adult worm pairs of *S. japonicum* were exposed to 1 - 5 μg/ml MQ, no death of worms was observed within three days [16]. They also revealed that when adult worms were exposed to 10 μg/ml MQ *in vitro* incubation, 22% (4/18) of male and 29% (4/14) of female worms died within one day. Thus, 10 μg/ml was considered to be the critical concentration of MQ to observe an anthelmintic effect on schistosomes. On the other hand, the present study showed that when adult worm pairs of *S. mansoni* were incubated with 10 μg/ml MQ within one day, all male and female worms died. This disparity indicates that MQ exerts a more powerful anthelmintic effect on the adult worms of *S. mansoni* than on those of *S. japonicum,* although the incubation medium used in experiments differed between Xiao *et al.* and the present study [16].

When adult worms were incubated with QN, all male and female worms survived for at least 14 days. Furthermore, QN did not inhibit the daily egg output of female worms. Keiser *et al.* reported that a single oral administration of QN resulted in worm burden reductions in the *S. mansoni*-mice model [15]. Thus, the antischistosomal activity of QN may be strengthened after the administration into mice.

Recently, Oliveira *et al.* showed that the 4-aminoquinoline derivatives CQ, AQ and PQ inhibited the formation of hemozoin [19], a detoxification product of free heme, in schistosomes and reported that the drugs exerted an effect on schistosomes. It is thought that free heme caused by the drugs might be responsible for damaging reproductive organs or killing worms. In the present study, AQ and PQ significantly reduced the survival times of adult worms and inhibited the egg output of adult female worms, respectively. At first, AQ caused remarkable blebs on the body of male and female worms in the early incubation period. Subsequently, male and female worms were swollen, sticky, and finally died. Although the morphological alterations of male and female worms caused by PQ were similar to those caused by AQ, the appearance of the morphological alterations in worms was more delayed in the PQ group than in the AQ group. On the other hand, while CQ did not kill male or female worms during the 14-day incubation, it exerted an inhibitory effect on the egg output of *S. mansoni* female worms. The present study demonstrated the strong antischistosomal activity of AQ during *in vitro* incubation. However, Keiser *et al.* reported that a single oral administration of AQ 400 mg/kg produced no antischistosomal activity in *S. mansoni*-infected mice [15]. Therefore, the *in vitro* effect of AQ on adult worms might be reduced in the *S. mansoni*-mouse model, probably due mainly to the metabolism of the drug or the dose administrated to mice. After oral administration, AQ is rapidly and extensively metabolized to *N*-desethylamodiaquine [20]. This main metabolite is assumed to have little or no effect on schistosomes. In addition, the single oral dose of AQ 400 mg/kg used in the *S. mansoni*-mouse model might be insufficient to achieve the desired therapeutic effect on schistosomes [16].

The antischistosomal activity of Sf was not observed in the present study. This finding indicates that sulfamethoxypyrazine (SMPZ), another sulfa derivative structurally related to Sf, is also likely to exert little or no effect on *S. mansoni.* On the other hand, Py, which is currently used for the treatment of malaria in combination with SMPZ, reduced the egg output of paired female worms in *in vitro* incubation. These results suggest that the antischistosomal activity of combination therapies of ART and SMPZ/Py is due mainly to the synergistic effect of ART with Py [12-14].

Our previous study showed that female worms were more susceptible to ART than male worms [8]. Furthermore, the present study showed that female worms were more susceptible to AQ and PQ than male worms (Table 2). However, no significant difference in the survival time of male and female worms was observed in the MQ group. In addition, Xiao *et al.* reported that the survival time of female worms of *S. japonicum* was similar to that of male worms during *in vitro* incubation with MQ [16]. It is difficult to conclude, therefore, that female schistosomes are more susceptible to all current antimalarial drugs than male schistosomes.

Some field trials have demonstrated the high antischistosomal effect of combination therapies with ART and current antimalarial drugs, indicating that ART exhibits the antischistosomal effect with current antimalarial drugs synergistically [12, 14, 21]. As a result, the antischistosomal effect of AQ, PQ, CQ and Py are noteworthy when these drugs are used for the treatment of malaria in schistosomiasis endemic areas. The present findings are interesting and useful to evaluate the effect of ACT on schistosomiasis. The ACT accompanied by AQ or SMPZ/Py [22] as the first-line treatment for malaria can be expected to provide additional benefits for schistosomiasis control. However, since ACT combined with current antimalarial drugs is prone to adverse effects [23, 24], the use of these drugs for the treatment of schistosomiasis should be carried out cautiously.

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