

## In vitro effects of amodiaquine on paired *Schistosoma mansoni* adult worms at concentrations of less than 5 µg/mL

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*In this study, the in vitro effects of amodiaquine (AQ) monotherapy on the egg output of paired adult Schistosoma mansoni worms and their survival during in vitro culture were assessed. In addition, the gross morphological alterations of male and female worms caused by AQ were visually observed under a dissecting microscope. AQ significantly reduced the daily egg output of paired adult S. mansoni worms following incubation for 14 days at 1-5 µg/mL, but not at 0.5 µg/mL, compared with the control group. AQ also reduced the survival of male and female worms at concentrations of 2 and 5 µg/mL, respectively. Moreover, exposure to 5 µg/mL AQ caused severe swelling and/or localisation of black content in the body of all male and female worms within one or two days of incubation; subsequently, shrinkage in the male worms and elongation in the female worms were observed. The initial morphological alterations caused by AQ occurred along the intestinal tract of the male and female worms. To our knowledge, this is the first study to report not only the efficacy of AQ at concentrations lower than 5 µg/mL on paired adult S. mansoni worms, but also the effects of AQ on the intestinal tracts of worms in in vitro culture.*

Key words: *Schistosoma mansoni* - amodiaquine - antimalarial drug - antischistosomal drug

Schistosomiasis is still a major public health problem in many tropical and subtropical countries. An estimated 779 million people are at risk for the disease and 207 million people are infected worldwide (Steinmann et al. 2006). Praziquantel (PZQ) has become the drug of choice for the treatment of human schistosomiasis (WHO 2002). However, field studies indicate the presence of PZQ-resistant schistosomes in Senegal and Egypt (Guisse et al. 1997, Ismail et al. 1999). Concerns regarding the emergence of a tolerant and/or resistant schistosomal strain are increasing. Thus, research related to the development of a new schistosomicidal drug is required.

Recently, Keiser et al. (2009) reported the interesting finding that mefloquine (MQ), one of the antimalarial drugs currently in use, reduced the worm burden in mice infected with *Schistosoma mansoni* and *Schistosoma japonicum*. Furthermore, Xiao et al. (2009) confirmed the in vitro effects of MQ against juvenile and adult worms of *S. japonicum*. On the other hand, the antischistosomal activity of amodiaquine (AQ), another antimalarial drug that is currently in use, was not observed in the *S. mansoni*-infected mice after a single oral administration (Keiser et al. 2009). Mitsui and Aoki (2010), however, demonstrated that not only MQ, but also other antimalarial drugs, such as AQ, chloroquine (CQ) and primaquine (PQ), exhibited in vitro antischistosomal activity at a concentration of 10 µg/mL. Subsequently, the in vitro antischistosomal activity of PQ was

confirmed at concentrations lower than 2 µg/mL (Holtfreter et al. 2011). Mitsui and Aoki (2010) observed that AQ exhibited higher in vitro antischistosomal activity than PQ at a concentration of 10 µg/mL and they cited AQ as a promising candidate for the treatment of schistosomiasis. Nevertheless, the in vitro antischistosomal activity of a relatively low concentration of AQ has not been investigated to date. This low concentration would correspond closely with the whole blood AQ concentration after administration of the drug at a dose of 10 mg/kg once daily for three days for the treatment of malaria (Winstanley et al. 1987, WHO 2001, Gitau et al. 2004).

The objective of this study was to evaluate the in vitro antischistosomal effects of AQ at concentrations of less than 5 µg/mL. The in vitro effects of the drug were examined with regard to the daily egg output of paired *S. mansoni* worms, survival and gross morphological alterations caused by the drug.

### MATERIALS AND METHODS

**Chemicals and media** - AQ·2HCl was purchased from MP Biomedicals Inc (Fountain Parkway Solon, Ohio, USA) and diluted in deionised water to a concentration of 5 mg/mL (free base) as a stock solution. The stock AQ solution 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, Oklahoma, USA), at concentrations of 0.5-5 µg/mL.

**Parasite strain** - A Puerto Rican strain of *S. mansoni* (NIH-Sm-PR-1) was routinely maintained by passage through Mongolian gerbils (*Meriones unguiculatus*; MGS/Sea, Kyudo Co, Ltd, Saga, Japan) and *Biomphalaria glabrata* snails (Newton's NIH Puerto Rican/Brazilian M-line) in the Animal Research Centre at the Institute of Tropical Medicine, Nagasaki University. At eight

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Received 25 April 2012

Accepted 2 October 2012

weeks post-infection, adult worms were collected by the perfusion technique as previously described (Smithers & Terry 1965) and washed twice with NCTC 135 medium.

The Animal Care and Use Committee, Nagasaki University, approved the experimental protocol and the experimental procedures were performed in accordance with the Guidelines for Animal Experimentation established by Nagasaki University.

**Incubation of paired *S. mansoni* adult worms with AQ** - The incubation of paired *S. mansoni* adult worms with AQ was conducted as previously described (Mitsui et al. 2009), except for the differences in the test drug and incubation period. Briefly, each pair of adult *S. mansoni* worms was preincubated for one day with 0.5 mL of NCTC 135 medium in a single well of a 24-well plate (Sumitomo Bakelite Co, Ltd, Osaka, Japan), in a 5% CO<sub>2</sub> incubator at a temperature of 37°C. Subsequently, 30 paired adult worms were randomly assigned to five groups: 0 (control), 0.5, 1, 2 or 5 µg/mL AQ. Then, each worm pair was transferred to a well containing 0.5 mL of NCTC 135 medium supplemented with 0, 0.5, 1, 2 or 5 µg/mL AQ. The plates were continuously incubated in the 5% CO<sub>2</sub> incubator at 37°C and the media alone or media supplemented with the drug was exchanged every day for 14 days. The number of eggs produced daily was counted and the morphological appearance of worms was also observed visually under a Nikon SMZ 800 stereoscopic microscope (Nikon Corporation, Tokyo, Japan).

The “dead or alive” status of adult worms was determined by the response of each worm to stimulation with a needle; that is, worms that failed to respond to needle stimulation were classified as “dead.” Photographs of worms treated with the drug were obtained using a digital Senamal C-mount camera (Microscope Network, Saitama, Japan) mounted on the stereoscopic microscope.

**Morphological investigation of intestinal tract alterations of male and female adult *S. mansoni* worms treated with AQ** - Twelve paired adult *S. mansoni* worms were incubated under the same conditions described above and divided into two groups. The six pairs of adult worms were incubated either with 0 (control) or 5 µg/mL AQ for two days, with daily changes of the media alone

or media supplemented with the drug. Then, the living male and female worms were individually mounted on a glass slide (76 x 26 x 0.9 ~1.2 mm; Matsunami Glass Industries, Ltd, Osaka, Japan) with two droplets of NCTC 135 medium, sandwiched with a coverslip (18 x 18 mm, N°1, Matsunami Glass Industries, Ltd, Osaka, Japan) and immediately observed under a stereoscopic microscope equipped with a digital camera.

**Data analysis** - The data were analysed using Epi-Info software (Centers for Disease Control and Prevention, Atlanta, Georgia, USA). The daily egg output per female adult worm was expressed as the arithmetic mean (± standard error). The Kruskal-Wallis or Mann-Whitney *U* test was used to compare daily egg output between the AQ-treated and control groups. A log-rank test was used to compare the survival time of worms between the AQ-treated and control groups.

## RESULTS

**Effect of AQ on the daily egg output of paired *S. mansoni* adult worms** - The in vitro effects of AQ on the daily egg output of paired adult worms at different concentrations are shown in Table I. The mean daily egg output during the one-day preincubation period in the control group did not differ significantly from that observed in the four AQ-treated groups. On the first day of exposure, AQ concentrations of 0.5, 1, 2 and 5 µg/mL did not significantly reduce the mean daily egg output compared with that of the control group. However, on days 2 and 3 after exposure of the worms to AQ, the mean daily egg output was significantly lower in the 1, 2 and 5 µg/mL AQ-treated groups than in the control group. Throughout the incubation period of seven days, the mean daily egg output of the 0.5 µg/mL AQ-treated group was similar to that of the control group.

**Effect of AQ on the survival of paired *S. mansoni* adult worms** - Table II shows the effect of continuous in vitro incubation with different concentrations of AQ on the survival of paired adult *S. mansoni* worms. None of the worms died in the control group during the 14-day incubation period. On the other hand, all of the male and female worms in the 5 µg/mL AQ group died within eight days. In the 2 µg/mL AQ group, four of the six

TABLE I  
Effect of amodiaquine on the daily egg output of six paired *Schistosoma mansoni* adult worms within seven days

Amodiaquine concentration (µg/mL)	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0 (control)	18.5 ± 8.7	64.3 ± 22.4	48.2 ± 11.7	33.2 ± 9.4	13.7 ± 7.2	1.0 ± 0.7	1.2 ± 1.2	2.8 ± 2.0
0.5	15.3 ± 3.4	42.7 ± 3.4	50.8 ± 5.2	38.5 ± 4.6	15.8 ± 4.8	4.0 ± 1.8	1.3 ± 0.7	0.2 ± 2.0
1	16.0 ± 6.5	15.5 ± 5.4	12.3 ± 4.3 <sup>a</sup>	1.3 ± 0.5 <sup>b</sup>	0.0	-	-	-
2	27.8 ± 18.5	30.0 ± 9.4	16.3 ± 5.5	3.7 ± 2.1 <sup>b</sup>	0.0	-	-	-
5	32.8 ± 8.2	18.0 ± 3.2	0.3 ± 0.2 <sup>a</sup>	0.0	-	-	-	-

a:  $p < 0.05$ ; b:  $p < 0.01$ , compared with the control group. Statistical analysis by Mann-Whitney *U* test.

TABLE II  
Effect of amodiaquine on the survival of six paired  
*Schistosoma mansoni* adult worms within 14 days

Amodiaquine concentration ( $\mu\text{g/mL}$ )	Number of dead worms	
	Male	Female
0 (control)	0/6	0/6
0.5	0/6	0/6
1	0/6	0/6
2	4/6 <sup>a</sup>	4/6 <sup>a</sup>
5	6/6 <sup>a</sup>	6/6 <sup>a</sup>

a: statistically significant vs. control at  $p < 0.01$  by a log-rank test.

males and four of the six females died before the end of the 14-day incubation period. No worm deaths were observed in the 0.5 or 1  $\mu\text{g/mL}$  AQ groups during the 14-day incubation period.

**Gross alterations of *S. mansoni* adult worms caused by AQ** - The gross morphological appearance of paired adult worms exposed to AQ was observed daily under a dissecting microscope. When paired adult worms were consecutively cultivated in media alone (control group), the black content in the body of all male worms gradually disappeared over a period of three days (Fig. 1A-C). The black content in the body of separated female worms also disappeared. Subsequently, both male and female worms shrank in size after a seven-day incubation period (Fig. 1D). On the other hand, when paired adult worms were exposed to 5  $\mu\text{g/mL}$  AQ for one or two days, severe swelling and localisation of black content appeared in the body of the male worms (Fig. 1F). On day 3 after exposure to 5  $\mu\text{g/mL}$  AQ, the male worms appeared slightly shrunken and exhibited visible localisation of black content, while the female worms were elongated

and exhibited a similar localisation of black content (Fig. 1G). On day 7 after exposure to 5  $\mu\text{g/mL}$  AQ, the male worms had shrunk further and simultaneously turned opaque with a damaged surface; in contrast, the female worms had elongated further and eroded and their surface had become sticky (Fig. 1H). Finally, all of the male and female worms died within eight days of incubation.

The morphological alterations observed (severe swelling and/or localisation of black content) also appeared in all of the male and female worms at low concentrations of 1 and 2  $\mu\text{g/mL}$  AQ during the incubation periods of five-seven and three-four days, respectively. At 0.5  $\mu\text{g/mL}$ , the specific morphological alterations appeared in all males as early as 10 days of incubation, but they were not observed in any of the females during the 14-day incubation period. Thus, the incubation time at which AQ caused the specific morphological alterations in male and female worms was closely related to the drug concentration used.

**Effects of AQ on the intestinal tracts of adult male and female *S. mansoni* worms** - Two days after the paired adult *S. mansoni* worms were continuously incubated in a drug-free medium (control group) or with 5  $\mu\text{g/mL}$  AQ, a small amount of black content lingering along the intestinal tract of males (Fig. 2A) was observed in the control group. Meanwhile, the intestinal tracts of the female worms appeared empty (Fig. 2B). In contrast, severe swelling and localisation of black content were visible in the male and female worms after two days of exposure to 5  $\mu\text{g/mL}$  AQ (Fig. 2C, D).

## DISCUSSION

Although monotherapy with artesunate (ART) in *Schistosoma haematobium*-infected children yields a low cure rate of 21% (Keiser et al. 2010), a high cure rate of 100% was obtained with the combination therapy of ART and AQ (Boulanger et al. 2007). These results suggest that AQ strongly exhibits antischistosomal activity. After oral administration at a dose of 10 mg/kg once daily for three days for the treatment of malaria in humans (WHO

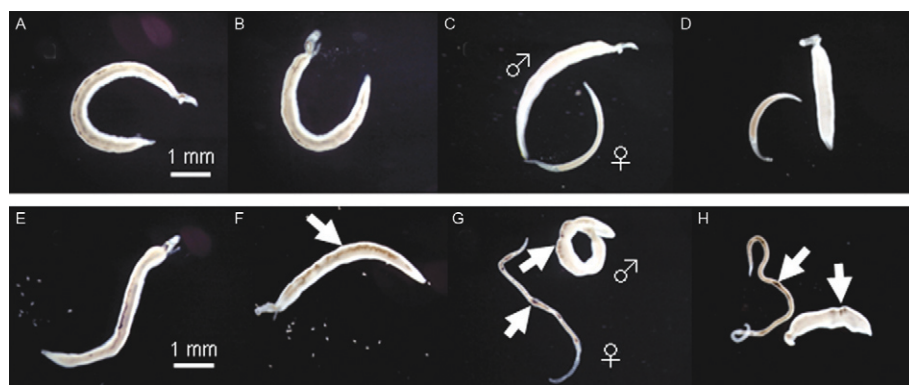


Fig. 1: effect of amodiaquine on gross morphological alterations in male and female adult worms of *Schistosoma mansoni*. Paired adult worms were incubated in media alone for zero (A), one (B), three (C) and seven (D) days (control group), while paired adult worms were incubated in media with 5  $\mu\text{g/mL}$  amodiaquine for zero (E), one (F), three (G) and seven (H) days. Arrows indicate localization of black content. The symbols ♂ and ♀ indicate male and female worms, respectively.



2001), the blood concentration of AQ and its major metabolite *N*-desethylamodiaquine (DEAQ) may reach a level close to 1 µg/mL (Winstanley et al. 1987, Gitau et al. 2004). Thus, it was surmised that AQ exerts antischistosomal activity at this concentration. However, the in vitro antischistosomal effect of AQ has not been further investigated at this concentration, although Mitsui and Aoki (2010) reported that AQ alone exerted an in vitro antischistosomal effect at a concentration of 10 µg/mL. An investigation of the antischistosomal activity of AQ at the low concentration of 1 µg/mL is very important to assess whether AQ alone exerts antischistosomal activity at the regular dose used for the treatment of malaria. Therefore, the aim of this study was to investigate the schistosomicidal effect of AQ on adult *S. mansoni* worms at the relatively low concentrations of 0.5–5 µg/mL.

When *S. mansoni* worm pairs were exposed to AQ at concentrations of 1, 2 and 5 µg/mL, the daily egg output was significantly lower than that of the control group after two or three days of incubation. Meanwhile, the daily egg output of the 0.5 µg/mL AQ group was similar to that of the control group. Thus, the minimum concentration of AQ that was effective against the daily egg output of *S. mansoni* worm pairs is presumed to lie between 0.5–1 µg/mL. Furthermore, the results of the present study showed that AQ reduced the survival time of adult worms at concentrations of 2 and 5 µg/mL during the 14-day incubation period. In addition, AQ caused specific morphological alterations in all of the male and female adult worms of *S. mansoni* at concentrations of 1, 2 and 5 µg/mL during the incubation periods of five–seven, three–four and one–two days, although these alterations were delayed in

the group treated with 0.5 µg/mL AQ. The direct effect of AQ on the daily egg output and morphological alterations of paired adult worms seemed to depend on the concentration and duration of exposure to the drug.

The effect of a drug is usually attributable to its pharmacokinetics in human subjects. The in vitro effects of AQ on adult *S. mansoni* worms demonstrated in the present study may not entirely reflect the in vivo effects of AQ. In fact, after oral administration, AQ is rapidly and predominantly metabolised to DEAQ (Churchill et al. 1985, Winstanley et al. 1987). Although both AQ and DEAQ exhibit antimalarial activity, the activity of DEAQ is approximately 3.5 times lower than that of AQ (Churchill et al. 1985, Childs et al. 1989). It follows, therefore, that the antischistosomal activity of DEAQ is also lower than that of AQ. Furthermore, during the regular treatment of malaria in an adult subject, the average concentration of DEAQ in whole blood reached  $0.56 \pm 0.07$  µg/mL at  $2.2 \pm 0.5$  h and gradually decreased to approximately 0.2 µg/mL (Winstanley et al. 1987) within 24 h. Thus, the standard dose of AQ for malaria (10 mg/kg of body weight, once daily for 3 days) is probably not sufficient to produce therapeutic effects in the treatment of schistosomiasis.

Schistosomes ingest the host red blood cells and degrade haemoglobin by converting toxic haem into haemozoin (Lawrence 1973, Brindley et al. 1997). Oliveira et al. (2004) showed that CQ, one of the 4-aminoquinoline derivatives similar in structure to AQ, exerted an effect on schistosomes by inhibiting the formation of haemozoin, a detoxification product of free haem. Similarly, it is thought that free haem produced by AQ may be responsible for damaging reproductive organs or killing worms. The free haem may also simultaneously damage the intestinal tract of male and female worms and suspend its rhythmic and peristaltic movement, resulting in severe swelling of the intestinal tract and/or a striking localisation of black content in the intestinal tract of worms as shown in Fig. 2C, D. These morphological alterations in male and female worms incubated with AQ had a very specific appearance. Although Holtfreter et al. (2011) did not report the effect of PQ on the intestinal tract of male and female worms, PQ, which is similar in structure to AQ, most likely also causes morphological alterations in the intestinal tract of male and female worms.

Some field trials have revealed a strong antischistosomal effect of combination therapies consisting of ART and current antimalarial drugs (Boulanger et al. 2007, Mohamed et al. 2009, Sissoko et al. 2009, Keiser et al. 2010), indicating that ART exhibits antischistosomal activities that are synergistic with current antimalarial drugs. Because ACT with AQ (WHO 2006) is currently recommended as a first-line treatment for malaria, the combination therapy using ART and AQ for the treatment of malaria in schistosomiasis-endemic areas may elicit an additional therapeutic effect on schistosomiasis. Thus, the present findings are noteworthy and can be expected to contribute to the evaluation of the therapeutic effects of the combination therapy on schistosomiasis in field trials. No serious side effects of AQ have been registered to date (D'Alessandro & ter Kuile 2006), but



Fig. 2: effect of amodiaquine on morphological alterations of the intestinal tract of male and female adult worms of *Schistosoma mansoni*. Paired adult male (A) and female (B) worms were incubated in media alone for two days (control group), while another pair of adult male (C) and female (D) worm was incubated with amodiaquine at a concentration of 5 µg/mL for two days. Each living worm was placed between a slide glass with medium and a glass coverslip and immediately observed. Arrows indicate the severe swelling of the intestinal tract of the male (♂) and localization of black content along the intestinal tract of the female (♀) worm.

minor side effects are relatively frequent in pregnant women treated with AQ alone. Thus, care should be exercised in the use of AQ alone or in combination with ART for the treatment of schistosomiasis.

#### ACKNOWLEDGEMENTS

To Dr Yoshiki Aoki, Graduate School of International Health Development, Nagasaki University, for assisting in the revision of the paper.

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