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Schistosoma mansoni: Schistosomicidal effect of mefloquine and primaquine *in vitro*

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ABSTRACT

We investigated the effects of the anti-malarials mefloquine and primaquine against the juvenile and adult life stages of *Schistosoma mansoni in vitro*. Cercariae were incubated with 0.5 μg/ml, 1 μg/ml and 2 μg/ml mefloquine or primaquine and with 1 μg/ml praziquantel for 12 h. Schistosomula, pre-adults and adults were incubated with 0.5 μg/ml, 1 μg/ml and 2 μg/ml mefloquine or primaquine and with 1 μg/ml praziquantel for 7 days. The viability status was classified as viable, damaged or dead and was checked every 3 h for cercariae and every 12 h for schistosomula, pre-adults and adults. Both, mefloquine and primaquine show time and dose-dependent schistosomicidal effects on the four life stages of *S. mansoni*. The promising *in vitro* effects on all stages of the blood fluke *S. mansoni* warrants further evaluation of both anti-malarials and their derivatives for their prophylactic and therapeutic values in early and late schistosomiasis in field trials.

1. Introduction

Schistosomiasis is one of the most prevalent tropical diseases worldwide. Upon penetration of the skin, the waterborne cercariae lose their tails and transform into schistosomula. The schistosomula then enter the venous system and reach the lungs, where they mature to pre-adults. Beginning 8–10 days after infection, the pre-adults reach the portal system, where they mature to adult males and females. Praziquantel is the treatment of choice and is effective against adult worms but much less so against juvenile stages like schistosomula, pre-adults and juvenile adults (Silva et al., 2003; Keiser et al., 2009).

The anti-malarial mefloquine, a synthetic analogue of quinine, has recently been shown to exhibit schistosomicidal activity in mice (Lademann et al., 2005; Van Nassauw et al., 2008; Keiser et al., 2009). While 150 mg/kg body weight, given 8 weeks after infection, had no effect on the worm burden, the number of eggs was reduced significantly (Van Nassauw et al., 2008). In another study, single oral doses of 200 and 400 mg/kg body weight, given 7 weeks after infection, resulted in worm burden reductions of 72% and 93%, respectively (Keiser et al., 2009). Recently, mefloquine was shown to exhibit anti-schistosomal properties against both, juvenile and adult parasites of *Schistosoma mansoni* and *Schistosoma japonicum in vitro* (Manneck et al., 2010; Xiao et al., 2009).

The anti-malarial primaquine, an 8-aminoquinoline, was found to affect lysosomal acidic vesicles of schistosomula that are involved in endocytosis and detoxification, if the parasite is damaged by drug treatment or immune attack (Carneiro-Santos et al., 2001).

Based on the promising effects of mefloquine and primaquine on schistosomes and the fact that both drugs are widely used in regions where both, malaria and schistosomiasis are endemic, this study was conducted to evaluate the effect of clinically achievable concentrations of the anti-malarials on cercariae, schistosomula, pre-adults and adults of *S. mansoni in vitro*.

2. Materials and methods

2.1. Animals

An African strain (Mozambique) of *S. mansoni*, maintained in *Biomphalaria glabrata* snails and female NMRI outbred mice (Harlan & Winkelman, Germany), was used throughout this work. Animals were kept in a 12 h day/night cycle. The experimental protocols were approved by the regional animal care and use committee.

2.2. Drugs

One hundred milligrams of mefloquine hydrochloride (Sigma, Germany) were dissolved in 2.5 ml dimethylsulfoxide (DMSO; Sig-

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ma) and then diluted in 47.5 ml *aqua ad injectabilia* (Braun, Germany) to obtain a stock solution of 2 mg/ml. Further dilutions were made in schistosomula or adult culture medium. One gram primaquine bisphosphate (Aldrich, Germany) was dissolved in 16.4 ml *aqua ad injectabilia* to prepare a stock solution of 60.98 mg/ml. Further dilutions were made in *aqua ad injectabilia*. One gram praziquantel (Sigma) was dissolved in 11 ml DMSO to obtain a stock solution of 90.9 mg/ml. Further dilutions were made in *aqua ad injectabilia*.

2.3. Drug application

Stock solutions of mefloquine and primaquine were diluted to reach final concentrations of 0.5, 1 and 2 μg/ml in the cavities of 12 well plates filled with a final volume of 3 ml schistosomula culture medium for schistosomula or 3 ml adult culture medium for pre-adults and in the cavities of six well plates filled with a final volume of 4 ml filtered aquarium water for cercariae or 5 ml adult culture medium for adults. DMSO was present in a maximum concentration of 0.005% in our experiments. Since DMSO is known to be cytotoxic at higher concentrations, we tested DMSO at concentrations from 0.005% to 1% and found no damaging effects on any life stages. Unexposed stages were used as negative controls throughout the experiments. As positive controls, cercariae, schistosomula, pre-adults and adults were incubated with 1 μg/ml praziquantel as described above. The chosen mefloquine, primaquine and praziquantel concentrations reflect the average serum level during chemotherapy and prophylaxis, respectively (Karbwang and White, 1990; Roche, 2009; Kollaritsch et al., 2000; Elmes et al., 2006; Singhasivanon et al., 1991; Cioli and Pica-Mattoccia, 2003). All experiments were performed in duplicates.

2.4. Reagents for cell culture and parasite preparation

RPMI-1640 culture medium with 13.3 μM phenol red and 2.05 mM L-glutamine and foetal calf serum (FCS) were obtained from GIBCO (Germany). FCS was heat inactivated at 56 °C for 30 min before usage. Penicillin G (benzylpenicillin), streptomycin sulfate, heparin sodium salt, L-arginine and D-glucose were obtained from Sigma (Germany).

2.5. Preparation of cercariae

Cercariae were obtained from infected *B. glabrata* snails after exposure to light for about 90 min. Infectious larvae were counted in one ml of the cercarial solution (portions of 100 μl) stained with iodide with the light microscope at 4-fold magnification. Afterwards one ml of the cercarial solution was added into each well. It was assumed that approximately the same numbers of cercariae were present in all examined wells.

2.6. Preparation of schistosomula

Schistosomula were transformed from cercariae by penetration of dissected shaved abdominal mouse skin of four 12-week-old NMRI mice as previously described (Clegg and Smithers, 1972). Thirty to seventy schistosomula were then cultured in each cavity of 12 well plates filled with schistosomula culture medium (RPMI-1640, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μM L-arginine, 2 μM D-glucose and 10% FCS) for 24 h at 37 °C and 5% CO_2. The drugs were then added as described above.

2.7. Preparation of pre-adults

Eight four- to eight-week-old NMRI mice were infected with *S. mansoni* by soaking in a 50 ml water bath containing 500–1500 cercariae for 90 min. The mice were sacrificed 6 days after infection and pre-adults were recovered from the lungs as previously described (Miller and Wilson, 1978). Fifty to seventy pre-adults were cultivated per cavity of 12 well plates in adult culture medium (RPMI-1640 medium, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS) for 24 h at 37 °C and 5% CO_2 (El-Ridi et al., 1997). The drugs were then added as described above.

2.8. Preparation of adults

Five four-to-eight-week old NMRI mice were infected with 300 cercariae of *S. mansoni* as described above and sacrificed 70 days after infection. Adult worms were recovered from the portal veins as previously described (Duvall and DeWitt, 1967). Five to seven adult worms were cultured per cavity of 6 well plates containing adult culture medium at 37 °C and 5% CO_2 for 24 h followed by drug application.

2.9. Mobility and viability

Since cercariae are known to lose infectivity rapidly after 12 h (Löscher et al., 2000), mobility and viability of the infectious larvae were observed for 12 h at 3-h intervals. Unaffected free swimming larvae were detected with a loupe with 4-fold magnification (Eschenbach, Germany). Immobile and dead cercariae at the bottom of the well were detected with an inverse light microscope (Nikon CK40) with 100-fold magnification. Schistosomula, pre-adults and adults were observed at 12-h intervals for seven days with an inverse light microscope using 100-fold magnification for schistosomula and pre-adults and 40-fold magnification for adults.

2.10. Statistical analysis

Effects of the substances were analyzed with respect to the viability status. Viability was assessed in the categories unaffected, damaged or dead at 12 h for cercariae, and at 168 h for schistosomula, pre-adults and adults. The microscopical evaluation was not done in a blinded manner. The Mantel–Haenszel chi square test and the chi square test were used to assess differences between groups. Statistical analysis was performed with statistical software SAS 9.1 (SAS Institute Inc, Carry, NC, USA). A p-value < 0.05 was considered to be statistically significant.

3. Results

The effect of mefloquine and primaquine on viability of cercariae, schistosomula, pre-adults and adults of *S. mansoni* was classified into the categories unaffected, damaged and dead by the parameters presented in table 1. Both, mefloquine and primaquine were found to have significant schistosomicidal effects on cercariae after 12 h and on schistosomula, pre-adults and adults after 168 h, that were dependent from drug concentrations (Figs. 1 and 2; $p < 0.0001$).

3.1. Mefloquine

3.1.1. Cercariae

At 2 μg/ml mefloquine all cercariae were damaged after 3 h and dead after 9 h. At 1 μg/ml mefloquine all cercariae were injured after 6 h and 113 of 575 cercariae (20%) were dead after 12 h (Fig. 1A). At 0.5 μg/ml mefloquine had no effect on cercariae compared to the unexposed control group after 12 h. The mefloquine-specific effect on cercariae was characterized by a strong vacuolisation of the cercarial head.