In vitro tegumental alterations on adult *Fasciola gigantica* caused by mefloquine

Hatem A. Shalaby · Amira H. El Namaky ·
Reem O. A. Kamel

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Abstract Emergence of drug-resistant *Fasciola* strains has drawn the attention of many authors to alternative drugs. The purpose of this study is to explore the in vitro effect of the antimalarial mefloquine against adult *Fasciola gigantica*. Light and scanning electron microscopic observations could be used to determine the target of the drug following 6 and 12 h of incubation in medium containing mefloquine at three different concentrations 10, 20 and 30 µg/mL, as morphological changes could be observed. These changes occurred in definite sequences in response to mefloquine, and were consisted of swelling, vacuolization that was later disrupted, leading to desquamation of the tegument, resulting in exposure and disruption of basal lamina and the dislodging of spines. It is concluded that mefloquine presented itself as a drug that might become important in trematode chemotherapy, with the tegument being an important drug target.

Keywords *Fasciola gigantica* · Mefloquine · Morphological changes · Body wall

Introduction

Tropical fasciolosis caused by infection with *Fasciola gigantica* is regarded as the most important single helminth infection of ruminants (Meaney et al. 2002) and still constitutes as one of the major economic and health problems.

It is considered an important limiting factor for livestock productivity (Khan et al. 2009). Yearly an estimated US\$ 2 billion are foregone due to weight loss, reduction in milk yield and fertility in production animals (Schweizer et al. 2005; McManus and Dalton 2006). In Egypt, the economical losses have been determined at 484.5 million LE per year according to the report of Central Organization of Mobilization and Computation, Cairo (2000), that figure is likely to be much higher now.

Fasciolosis could be partially controlled by periodic treatment of animals in endemic areas with drugs, among which triclabendazole (TCBZ) was reported to be highly effective (Overend and Bower 1995). A benzimidazole derivative, TCBZ is the drug of choice as it is safe and efficacious against both juvenile and adult flukes (Fairweather and Boray 1999) and has been marketed since 1983 as a veterinary drug (Fasinex®) (Keiser and Utzinger 2005). Various studies confirmed the occurrence of TCBZ resistance in sheep and cattle from different parts of the world as e.g., Australia, Ireland, The Netherlands, Spain and the UK (Keiser et al. 2005; Alvarez-Sanchez et al. 2006). There are no drugs of comparable activity currently available for the treatment and control of fasciolosis, hence there is a pressing need for new trematocidal drugs. In 2008, mefloquine, an amino alcohol antimalarial drug used widely in the treatment and prophylaxis of malaria for about 40 years, was reported to be effective in significant reduction of egg production in *Schistosoma mansoni*-infected mice after administration of a single dose of 150 mg/kg, but the drug had no effect on worm burden (Van Nassauw et al. 2008). Recently, mefloquine was shown to exhibit anti-schistosomal properties against both, juvenile and adult parasites of *S. mansoni* and *S. japonicum* in vitro (Xiao et al. 2009a; Manneck et al. 2010, 2011; Holtfreter et al. 2011). As the tegument is one of the main absorptive surfaces for the uptake of drugs by the fluke

H. A. Shalaby (✉) · A. H. El Namaky
Department of Parasitology and Animal Diseases, National
Research Center, Giza, Egypt
e-mail: shalaby85@gmail.com

R. O. A. Kamel
Department of Zoology, Girls College for Art, Science and
Education, Ain Shams University, Cairo, Egypt

(McKinstry et al. 2003), the present study was undertaken to evaluate changes in tegument of adult *F. gigantica* following exposure to mefloquine in vitro.

Materials and methods

Drug

Mefloquine, 4-quinolinemethanol derivative, (Lariam[®]) was obtained from Roche Products Pty Ltd (Australia).

Parasite

Adult *F. gigantica* were recovered from the bile ducts of buffalo, slaughtered in Cairo abattoir, under sterile conditions in a laminar flow cabinet and washed in several changes of warm (37 °C), sterile complete RPMI 1640 culture medium containing antibiotics (penicillin, 50 IU/mL; streptomycin, 50 µg/mL).

Effect of mefloquine on adult flukes

The flukes were subsequently transferred to fresh culture medium containing 50 % (v/v) heat denatured rabbit serum, 2 % (v/v) rabbit red blood cells; as recommended by Ibarra and Jenkins (1984), and mefloquine at three different concentrations 10, 20 and 30 µg/mL. Where, stock solution of mefloquine at 10 mg/mL was prepared with 60 % (v/v) dimethyl sulphoxide (DMSO) for immediate use. Then the whole flukes were incubated for 6 and 12 h at 37 °C in an atmosphere of 5 % CO₂. Solvent control flukes were incubated for 12 h in RPMI 1640 culture medium containing 0.1 % (v/v) DMSO; corresponded to maximum solvent concentration. Normal control at 0 h was fixed immediately following the initial washing. Six flukes were examined for each concentration.

Light microscopy

Following incubation, the adult flukes were cut into small, 5 mm pieces before being fixed in 10 % buffered formol saline, dehydrated, cleared and embedded in paraffin blocks. Paraffin sections of 5 µ thickness were prepared and stained by Haematoxylin and Eosin (H&E) according to the method of Bancroft et al. (1996). The body wall of adult flukes was examined and photographed using an Olympus CX41 microscope.

Scanning electron microscopy (SEM)

Following 12 h of incubation with 30 µg/mL mefloquine, the anterior end (including mid body region) of adult flukes

was fixed intact for 12 h in a 3:1 mixture of 4 % (w/v) glutaraldehyde in 0.12 M Millonig's buffer, pH 7.4 and 1 % aqueous osmium tetroxide. After this, the specimens were processed for SEM following a method previously reported by Shalaby et al. (2009).

Results

Light microscopic observations of the tegument cross section

Control flukes

The tegumental morphology of solvent control specimens appeared normal, and similar to fresh control specimens, when examined using light microscopy. A brief description of some of the important tegumental features was necessary to assess the changes resulting from mefloquine treatment. The tegument showed even cytoplasmic syncytial layer with numerous spines embedded throughout its matrix. The tegument rested on layer of connective tissue called reticular lamina, which connected the former to the underlying and deeply stained two muscular layers (Fig. 1a, b). Thus no abnormalities were noted following incubation in vitro.

Treated flukes

Following incubation for 6 h With 10 µg/mL mefloquine, tegumental swelling and furrows were seen as well as the tegument between the spines and also that covering the spines were severely swollen so that only the tips of the spines were protruded (Fig. 1c, d). These changes became more severe with spines appeared to be completely submerged by the extremely swollen tegument, with 20 µg/mL mefloquine. Besides, appearance of numerous vacuoles in the tegumental syncytium, and the tegument surrounding the spines was seen to be split (Fig. 1e, f). With 30 µg/mL mefloquine, the tegument appeared to be very vacuolated, particularly in the basal region and that covering the spines. The spines remained attached to the basal lamina in the region where the tegument remained (Fig. 1g) however,

Fig. 1 Light micrographs of the tegument cross section of adult *F. gigantica*. **a, b** Normal fresh flukes. **c–h** Following 6 h incubation with mefloquine. **c, d** With 10 µg/mL mefloquine. Note tegumental swelling and the spines appear to be surrounded by the tegument. **e, f** With 20 µg/mL mefloquine. Note appearance of numerous vacuoles in the tegumental syncytium (*black arrow*) and splitting the tegument surrounding the spines. **g, h** With 30 µg/mL mefloquine. In this specimen, the tegument appears to be very vacuolated, particularly in the basal region and that covering the spines (*black arrow*). Other area shows spine dislodged from its socket (SS). *T* tegument, *S* spine, *M* muscular layer, *P* parenchyma

Fig. 2 Light micrographs of the tegument cross section of adult *F. gigantica* following 12 h incubation with mefloquine. **a, b** With 10 µg/mL mefloquine. The tegument appears to be swollen and shows extensive cracking. **c, d** With 20 µg/mL mefloquine. Note appearance of small isolated areas of tegumental splitting (black arrow). **e, f** With

30 µg/mL mefloquine. Patches of tegument has been removed (black arrow). In some specimens, the tegument appears to be completely sloughed with only the basal lamina (BL) remaining. T tegument, S spine, M muscular layer, P parenchyma

other areas showed spines dislodged from their sockets (Fig. 1h). Flooding was observed in the intercellular spaces between muscle bundles which appeared to be entirely separated from the surrounding tissue.

Following incubation for 12 h With 10 µg/mL mefloquine, disruption of the tegumental syncytium was evident and the tegument appeared to be very swollen and showed

extensive cracking towards the basal lamina so that the tegument surrounding the spines was seen to be sloughing away (Fig. 2a, b). With 20 µg/mL mefloquine, the tegumental disruption became more pronounced. It was in the form of small isolated areas of tegumental splitting, and sloughing of the tegument around the spine tips and, in some cases, complete spine loss from the tegument (Fig. 2c, d). With 30 µg/mL mefloquine, severe disruption

Fig. 3 Scanning electron micrographs (SEMs) of the anterior end of adult fluke including the mid-body region. **a, b** Normal fresh flukes. **a** SEM of the oral cone surface showing smooth oral sucker at its tip and the ventral sucker. **b** SEM of the tegument at mid-body region showing ridged tegument with numerous serrated spines. **c–f** Following 12 h incubation with 30 µg/mL mefloquine. Massive loss of tegument occurs in both the oral cone and the mid-body region to

expose the underlying basal lamina and empty spine sockets (SS). The posterior mid-body region exhibits an interface (*white arrow*) marking the separation between an area, where the tegumental syncytium had sloughed away to reveal the basal lamina and another area, where the tegumental syncytium remained. OS oral sucker, VS ventral sucker, C cirrus, S spine

was evident in the tegument of the fluke. Patches of tegument had been completely removed, exposing the basal lamina (Fig. 2e). In areas where the tegument remained, the tegument appeared to be very vacuolated. In some

specimens, extensive tegumental sloughing was observed, with only the basal lamina remaining and severe damage was observed in muscle bundles and parenchymal tissues (Fig. 2f). These observations implied that the most affected

target organ from the treatment with mefloquine was the tegument.

SEM observations following twelve hours incubation with 30 µg/mL mefloquine

The typical intact tegument surface of adult fluke exhibited microridges and grooves with numerous serrated spines that were most numerous on the anterior part especially around the oral and ventral suckers (Fig. 3a, b) and reduced in number on posterior part of the body. Abnormal changes were not observed on the tegumental surface of the solvent control flukes incubated up to 12 h in the medium containing 0.1 % DMSO. In contrast, the samples treated with mefloquine, at concentration 30 µg/mL at 12 h incubation showed severe and extensive disruption to the surface morphology, as illustrated in Fig. 3c–f. Massive loss of tegument occurred in all of the treated flukes, with the entire syncytial layer having been stripped off to expose the underlying basal lamina and empty spine sockets (Fig. 3e). This was true of both the oral cone and the mid-body region, although the sloughing was more severe in the anterior mid-body region than the posterior one. The latter exhibited a distinct boundary, or interface, marking the separation between an area, where the tegumental syncytium had sloughed away to reveal the basal lamina and another area, where the tegumental syncytium remained. Anterior to the interface, the spines were wholly removed from the tegumental syncytium, leaving behind empty spine sockets. Posterior to the interface, the tegumental syncytium appeared swollen and the spines appeared to be partially submerged (Fig. 3f).

Discussion

This preliminary study is the first to demonstrate the in vitro effects of mefloquine on the adult *F. gigantica*. Interestingly, mefloquine is the third antimalarial drug possessing fasciolicidal properties after artemisinins (artemether and artesunate) and trioxolanes (Keiser et al. 2006; Keiser and Morson 2008; Shalaby et al. 2009). This study indicated that mefloquine exhibited fasciolicidal activity in vitro and that the tegument of *F. gigantica* was a primary drug target. Differences in response to mefloquine action were observed, depending on the used concentration. Light and scanning electron microscopic observations could be used to determine the target of the drug as morphological changes could be observed. These changes occurred in definite sequences in response to mefloquine, and were consisted of swelling, vacuolization that was later disrupted, leading to desquamation of the tegument,

resulting in exposure and disruption of basal lamina and the dislodging of spines.

The tegument of *Fasciola* spp. had many of the characteristics of a transporting epithelium that was involved in ion and water controls. The swelling of tegumental syncytium indicated that disruption of the apical membrane and its associated ion pumps had led to perturbation of the fluke's osmoregulatory system and the influx of water (Devinea et al. 2012). The swelling of the basal infolds would facilitate the sloughing of the tegument as a whole, by causing the detachment of the basal plasma membrane from the underlying basal lamina. This swelling was a particular feature of drug action with a number of flukicides as recently summarized (Devinea et al. 2012). Promising activities of the antimalarial mefloquine against biologically related trematodes, the schistosomes, had been described recently. When the adult schistosomes were exposed to mefloquine at concentrations of 20 and 30 µg/mL, the process of the drug against the worms was fast that over 50 % of the worms died within 4 h, and the remaining worms died within 24 h, demonstrating that mefloquine exhibited a direct killing effect on adult schistosomes in vitro (Xiao et al. 2009a). Besides, exposure of adult *S. japonicum* to mefloquine in vivo resulted in extensive damage of the worm's digestive system, tegument, musculature, parenchymal tissues, and reproductive system (Xiao et al. 2009b).

In this study, the rapid and clear alteration of the tegumental integrity after contact with mefloquine began with vacuolization of the basal tegumental layer and vacuoles formed in the syncytium. Vacuolization might therefore be regarded as a direct and primary effect of mefloquine on the tegument of adult *F. gigantica*. Similar vacuolization of the tegument had been described in a number of anthelmintic-treated schistosomes (Xiao et al. 2002), *Opisthorchis viverrini* (Apinhasamit and Sobhon 1996), and also in *F. hepatica* (Halferty et al. 2009). Vacuolization was evident due to swelling of the basal infolds leading to sloughing of the syncytium. Once the tegument had been removed, the drug would be able to penetrate deeper into the internal tissues of the fluke and caused more widespread disruption, this was noticed at 12 h of incubation with 30 µg/mL mefloquine and was accompanied with loss of spines. Such tegumental damage of adult fluke, in infected host, might release fluke body surface antigen which interact with host specific antibody in the blood to promote fast and severe immunopathological response to deteriorate and kill the fluke. However, the results revealed no vacuole protruded on the tegumental surface examined by SEM. Similar pattern of schistosomes tegumental damage induced by mefloquine was observed by Xiao et al. (2010). He reported that histopathological observation on the worms shifted to the liver of mice treated with

mefloquine, vacuoles were only observed beneath the tegument, and no other vacuoles protruded from the tegumental surface, which was quite different from artemether. Other SEM studies on *S. mansoni* following mefloquine administration in vivo and incubation with mefloquine in vitro showed that the tegument seemed to be a main drug target as extensive tegumental disruption was observed (Manneck et al. 2010). Moreover, a study on *O. viverrini* revealed similar tegumental alterations as sloughing, which occurred rapidly following incubation of the liver flukes with mefloquine in vitro (Keiser et al. 2009).

In conclusion, the present study showed promising fasciolicidal properties of mefloquine and that the tegument of trematodes is subject to severe disruption. Whether this is due to direct uptake by the tegument or an indirect effect via disruption of the gut, remains to be determined. Thus, the relative contributions of the tegument and gut to the uptake of mefloquine and its relationship to morphological changes in the tissues of the fluke need to be explored further.

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