Morphological effects and tegumental alterations induced by mefloquine on schistosomula and adult flukes of *Schistosoma mansoni*

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SUMMARY

There is a pressing need to develop novel anti-schistosomal drugs, as current treatment relies largely on praziquantel (PZQ). To further strengthen current evidence of the anti-schistosomal properties of mefloquine (MQ), we studied the temporal effect of this compound *in vitro* and *in vivo*, and examined alterations on the tegumental surface of schistosomula and adults of *S. mansoni* by means of scanning electron microscopy (SEM). Schistosomula and adults were each incubated *in vitro* using MQ over a wide concentration range (1–100 μg/ml). In addition, mice infected with adult *S. mansoni* were treated with a single oral dose of 400 mg/kg MQ, and worms were recovered 24, 48, 72, 96 and 120 h following treatment. MQ showed a rapid onset of action on schistosomula *in vitro*; 100 and 75 μg/ml of MQ killed schistosomula immediately; the minimal lethal and effective concentrations of MQ on schistosomula after 1 h were 25 and 5 μg/ml, respectively. Adult worms incubated with 100 and 10 μg/ml of MQ were dead after 1 h and 24 h of incubation, respectively. A hepatic shift of adult schistosomes was observed in mice already 24 h after treatment, and 120 h following treatment >98% of all worms had translocated to the liver. SEM observations revealed extensive tegumental destruction, including blebbing, shrinking and sloughing, particularly following *in vitro* incubation and on the tegument of female worms.

Key words: *Schistosoma mansoni*, schistosomiasis, mefloquine, *in vitro*, *in vivo*, scanning electron microscopy.

INTRODUCTION

Schistosomiasis is a neglected tropical disease (NTD) caused by blood flukes of the genus *Schistosoma*. *Schistosoma haematobium*, *S. japonicum* and *S. mansoni* are the 3 main species parasitizing humans (Utzinger and Keiser, 2004; Gryseels *et al*. 2006). It has been estimated that there are more than 207 million people infected worldwide, with 779 million people at risk of infection (Steinmann *et al*. 2006). The annual mortality rate in sub-Saharan Africa is estimated to be as high as 280 000 (van der Werf *et al*. 2003). Estimates of the global burden of schistosomiasis range from 1·7 to 4·5 million disability-adjusted life years (DALYs) (WHO, 2002, 2004); the latter figure is likely to underestimate the true burden, as it does not consider chronic disability (Hotez *et al*. 2006). Revised estimates for schistosomiasis are at 3–70 million DALYs (King and Dangerfield-Cha, 2008).

Despite schistosomiasis being one of the most prevalent and debilitating NTDs, a devoted drug discovery and development programme does not exist (Ridley and Kita, 2007). Since the late 1970s, the treatment and control of schistosomiasis relies on a single drug, namely praziquantel (PZQ) (Gönnert and Andrews, 1977; Cioli and Pica-Mattoccia, 2003; Utzinger and Keiser, 2004; Doenhoff *et al*. 2008). However, the heavy reliance and application of PZQ within the frame of large-scale preventive chemotherapy treatment programmes might select for drug-resistant parasites (Botros and Bennett, 2007; Caffrey, 2007). In addition, PZQ has only moderate activity against juvenile worms, which is an important deficiency in its spectrum of activity, as it requires administration of a second PZQ dose after a short interval to eliminate the parasites that have since matured (Doenhoff *et al*. 2008).

Nonetheless, in spite of the lack of dedicated product development partnerships for helminth diseases, a number of compounds with interesting anti-schistosomal properties have been identified by various research groups in recent years; for example, peroxidic compounds (the semi-synthetic artemisinins and the synthetic trioxolanes (OZs)), 4-phenyl-1,2,5-oxadiazole-3-carbonitrile-2-oxide and the cysteine protease inhibitor K11777 (Abdulla *et al*. 2007; Keiser and Utzinger, 2007; Utzinger *et al*. 2007; Xiao *et al*. 2007; Sayed *et al*. 2008; Caffrey *et al*. 2009). An important finding was that mefloquine (MQ), an arylamino alcohol (4-quinoline-methanol), developed by the Walter Reed Institute of Research

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in the early 1970s (Sweeney, 1981) and now marketed for the prophylaxis and treatment of malaria, showed promising anti-schistosomal activity in mice. Single oral doses of 200–400 mg/kg to mice infected with either juvenile or adult stages of *S. mansoni* or *S. japonicum* resulted in high total or even complete reductions in female worm burdens (Keiser *et al.* 2009). Treatment with a lower dose of 150 mg/kg MQ revealed a significantly reduced egg production in *S. mansoni*-infected mice (Van Nassauw *et al.* 2008).

The aim of the present study was to further strengthen the current evidence base of the anti-schistosomal properties of MQ and to gain a first insight into the potential mode of action of the drug. The dose-response relationships were studied *in vitro*. The temporal drug effect was monitored *in vitro* and *in vivo*. In addition, alterations in the tegumental surface of schistosomula and adult flukes were studied by means of scanning electron microscopy (SEM).

MATERIALS AND METHODS

Experimental animals and parasites

All experiments were carried out at the Swiss Tropical Institute (Basel, Switzerland), in accordance with Swiss national animal welfare regulations. Female NMRI mice ($n = 40$, age = 3 weeks, weight = 25 g) were purchased from Harlan (Horst, The Netherlands).

Cercariae of *S. mansoni* (Liberian strain) were obtained from infected intermediate host snails (*Biomphalaria glabrata*) maintained according to standard procedures (Keiser *et al.* 2009).

Drugs

Mefloquine ((*RS*)-(±)-α-(*2-Piperidinyl*)-*2,8-bis*(*trifluormethyl*)-*4-quinolinemethanol hydrochloride*, MW = 414.81 g/mol) (MQ) was kindly provided by Mepha AG (Aesch, Switzerland). For *in vitro* studies, stock solutions of MQ (10 mg/ml) were prepared in dimethyl sulfoxide (DMSO) (Fluka, Buchs, Switzerland). For *in vivo* studies, MQ (40 mg/ml free base) was suspended in 7% (v/v) Tween 80 and 3% ethanol shortly before oral administration to mice.

In vitro *studies on schistosomula*

Cercariae of *S. mansoni* were mechanically transformed into schistosomula (Ramalho-Pinto *et al.* 1974). Briefly, 50 ml of an ice-cold cercarial suspension were centrifuged and the packed volume of cercariae was re-suspended in 2 ml of Minimal Essential Medium (MEM) (Eagle, 1959) and vortexed for 2 min to trigger tail loss. For the isolation of cercarial bodies, cold Hank's Basal Salt Solution (HBSS) was added to the cercarial suspension up to a volume of 7 ml. After cooling on ice for 15 min, the tail-rich supernatant was decanted and the sedimented bodies were re-suspended in 7 ml of cold HBSS. This procedure was repeated twice. The schistosomula suspension was kept in Basch culture medium (Basch, 1981) in an incubator at 37 °C in an atmosphere of air, (95%) + CO_2 (5%) for 1–3 h until use.

To study the temporal drug effect and the concentration-response relationships, schistosomula were incubated in 24-well plates (Costar), with 500–1000 schistosomula per well, in the presence of different concentrations of MQ. Each well contained 1 ml of Basch medium supplemented with 5% heat-inactivated foetal calf serum (iFCS) and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, USA) and MQ at 100, 75, 50, 25, 15, 10, 5 or 1 μg/ml, respectively. Schistosomula incubated with 1% DMSO, the highest concentration of drug solvent, served as controls. The parasites were kept in an incubator at 37 °C in an atmosphere of air, (95%) + CO_2 (5%) for up to 96 h.

Immediately after adding (0·125), 0·5, 1, 6, 17, 24, 48, 72 and 96 h after incubation with MQ, all schistosomula were observed under a dissecting microscope and the effect of MQ was assessed, with an emphasis on changes in worm motor activity, morphological/tegumental alterations and the occurrence of death. These phenotypic changes were scored using a viability scale of 0–3: (3 = totally vital, normally active, no morphological changes, 2 = slowed activity, first morphological changes and granularity visible, 1 = minimal activity, severe morphological changes and granularity, 0 = all worms dead, severe granularity) based on standard procedures for compound screening at WHO-TDR (Ramirez *et al.* 2007) and the UCSF Sandler Center (Caffrey *et al.* 2009). The minimal lethal concentration (MLC), which is the minimum concentration needed to kill all schistosomula and the minimal effective concentration (MEC), which is the minimum concentration needed to observe any change in viability or morphology of schistosomula were determined after 1 h. All experiments were carried out in duplicate and were repeated at least 3 times.

For SEM studies, samples of schistosomula incubated with 3 different concentrations of MQ (100, 10 and 1 μg/ml) were taken at different time-points (after 0·5, 1, 6, 24, 72 and 96 h). Specimens were fixed with 2·5% (v/v) glutaraldehyde in a phosphate-buffered saline (PBS) (pH 7·4) for 24 h at 22–24 °C (room temperature). After rinsing 3 times with PBS, the specimens were stored in the same buffer at 4 °C until use. Before SEM examination, the flukes were washed twice with double-distilled water, dehydrated in ascending ethanol concentrations and then air-dried. Schistosomula were placed on aluminium stubs, sputter coated with 20 nm gold particles and

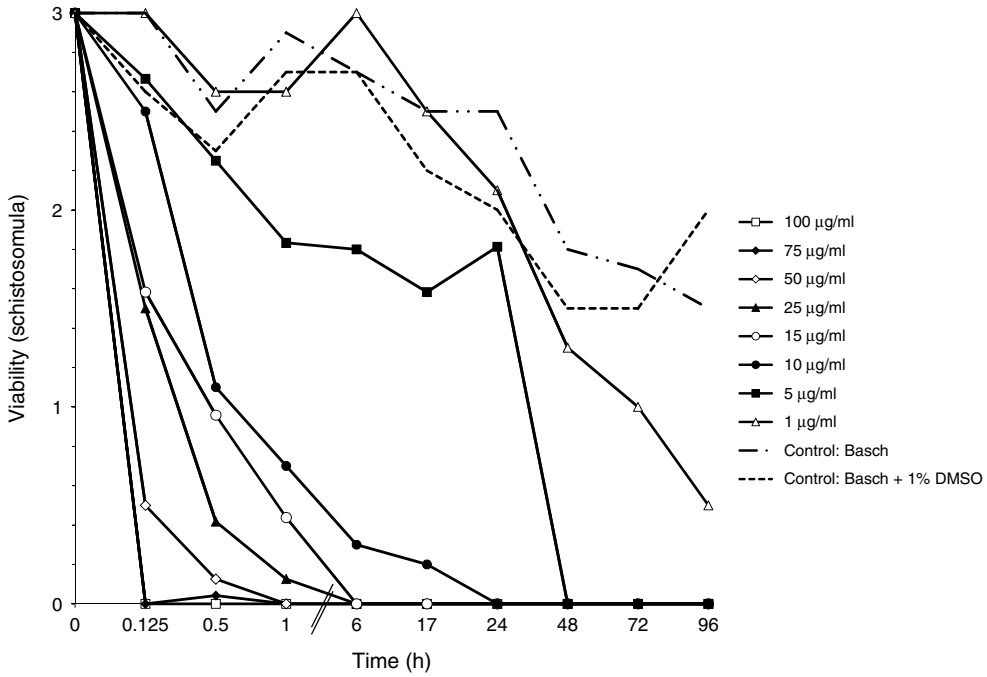

Fig. 1. Effect of different concentrations of MQ on the viability of *Schistosoma mansoni* schistosomula. Mean values of viability using a viability score. Numbers derived from a minimum of 6 experiments (500–1000 schistosomula per experiment were scored at each concentration). Open squares: 100 μg/ml; filled diamonds: 75 μg/ml; open diamonds: 50 μg/ml; filled triangles: 25 μg/ml; open circles: 15 μg/ml; filled circles: 10 μg/ml; filled squares: 5 μg/ml; open triangles: 1 μg/ml; dashed and dotted line: Control (Basch medium); dashed line: Control (Basch medium + 1% DMSO).

observed in a high-resolution SEM (Phillips XL30 ESEM) at an accelerating voltage of 5 kV.

In vitro *studies of adult schistosomes*

NMRI mice were infected subcutaneously with ~80 cercariae of *S. mansoni* and euthanized with CO_2 after 56 days. All adult schistosomes were collected from the hepatic portal system and mesenteric veins and removed from the liver by perfusion with ice-cold PBS supplemented with heparin (Yolles *et al.* 1947; Smithers and Terry, 1965). Schistosomes were incubated in 24-well plates (Costar), placing 2 male and 2 female worms in each well. Individual wells contained RPMI 1640 culture medium (Invitrogen, Carlsbad, USA) supplemented with 5% heat-inactivated calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, USA). Schistosomes were incubated in the presence of 100, 10 and 1 μg/ml MQ at 37 °C in an atmosphere of air (95%) + CO_2 (5%). Medium without and medium with 1% DMSO (the highest concentration of drug solvent used) served as controls. Before incubation with MQ and immediately after incubation (0·125), 1, 6, 24, 48 and 72 h with MQ, all *S. mansoni* were observed under a dissecting microscope. The effect of the drug was assessed with an emphasis on changes in worm motor activity, morphological/tegumental changes and death of worms. Death was defined as no movement observed for at least 2 min of examination and no movement at the other observation time-points. All experiments were carried out in duplicate and were repeated at least 3 times. For SEM examination, the worms were prepared as described above, with the exception that the worms were critical-point dried (Bomar SPC-900; Tacoma USA).

In vivo *studies*

NMRI mice were infected as described above. At 7–8 weeks after infection, mice were treated with a single oral dose of 400 mg/kg MQ, the most effective dose as established in our previous work (Keiser *et al.* 2009). At each time-point after treatment (24, 48, 72, 96 and 120 h), 3–6 mice were killed by cervical dislocation and the mesentery and the liver were collected. Schistosomes were removed from the mesenteric veins, sexed and counted using a binocular microscope. The liver was flattened and examined for the presence of worms. The distribution of schistosomes in the liver and mesenteric veins was recorded. Schistosomes recovered from the mesenteric veins were further examined, and changes in worm motor activity, morphological/tegumental changes and death were documented. Worms were then prepared for SEM examination.

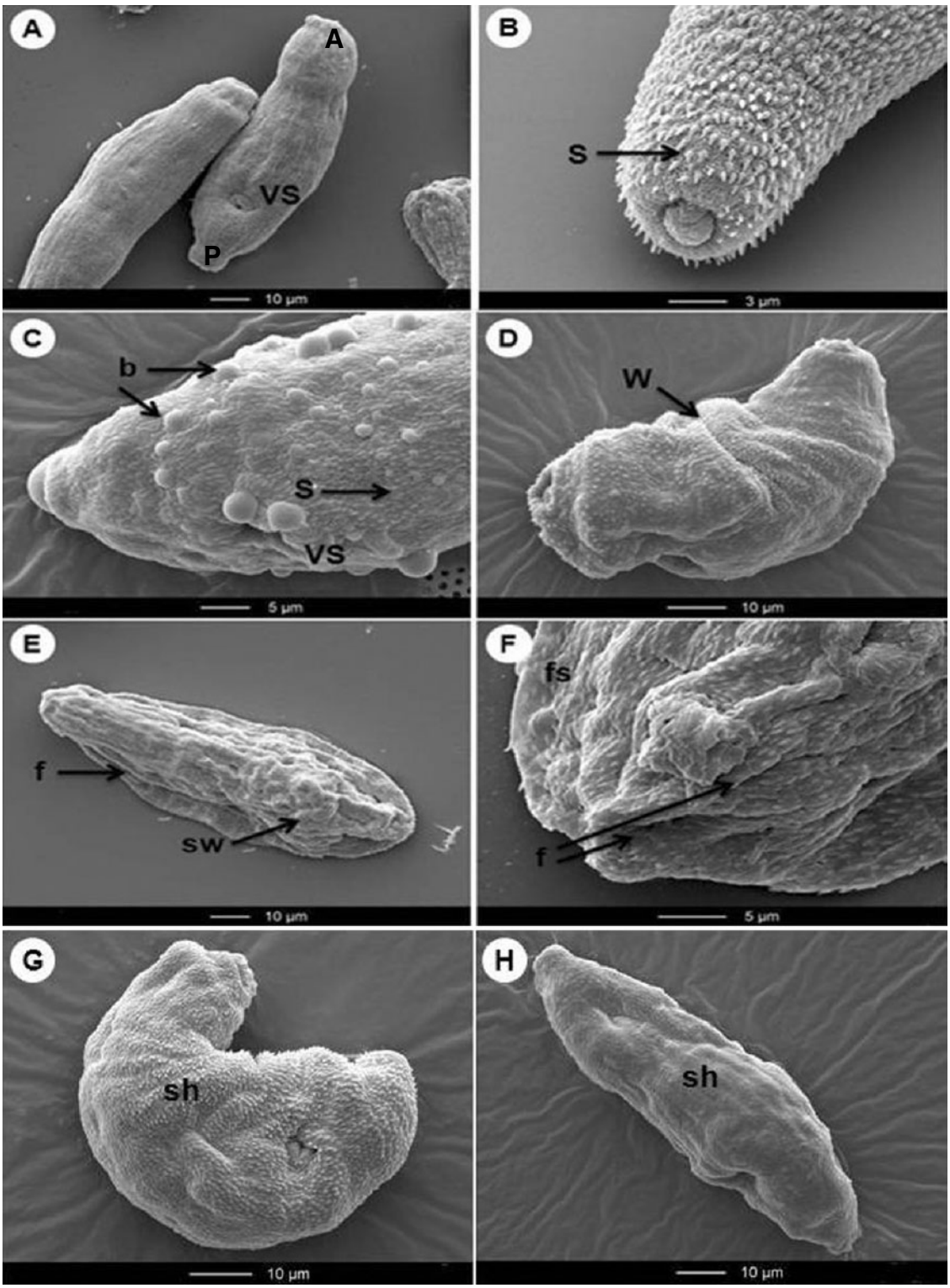

Fig. 2. SEM observations of *Schistosoma mansoni* schistosomula after *in vitro* incubation. (A) Control, showing the ventral sucker (VS) (=acetabulum), the anterior end (A) and the posterior end (P). (B) Control. Posterior region with clearly defined tegumental spines (S). (C) Thirty min after incubation with MQ (100 µg/ml). Shortened spines (S) and blebbing (b) near the ventral sucker (VS) visible. (D) Thirty min after incubation with MQ (10 µg/ml). Contraction and formation of wrinkles (W) visible. (E) Six h after incubation with MQ (10 µg/ml). A shrunken body with swelling (sw) and many furrows (f) observed. (F) Six h after incubation with MQ (10 µg/ml). The tegument shows deep furrows (f) and flattened spines (fs). (G) Twenty-four h after incubation with of MQ (1 µg/ml). Shrunken (sh) tegument and bending of the body. (H) Seventy-two h after incubation with MQ (1 µg/ml). A shrunken (sh) tegument visible.

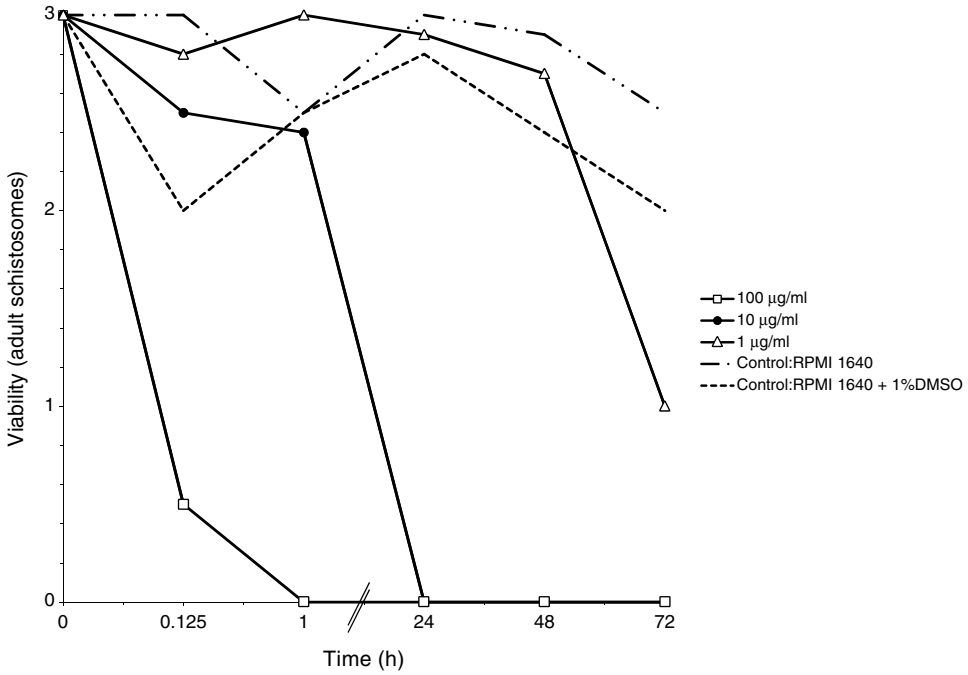

Fig. 3. Effect of different concentrations of MQ on the viability of adult worms of *Schistosoma mansoni*. Mean values of viability using a viability score. Numbers derived from a minimum of 6 experiments (in total 24 worms were scored at each concentration). Open squares: 100 μg/ml; filled circles: 10 μg/ml; open triangles: 1 μg/ml; dashed and dotted line: Control (RPMI 1640); dashed line: Control (RPMI 1640 + 1% DMSO).

RESULTS

In vitro *studies of schistosomula – dissecting microscopic and SEM evaluations*

The effect of incubation with different concentrations of MQ on the viability of schistosomula for up to 96 h is depicted in Fig. 1. In the absence of the drug, schistosomula showed normal viability without any morphological change for up to 24 h. Slight morphological changes such as granularity, a shorter body length with a falcate shape and reduced activity, were observed after 48 h of incubation. Schistosomula were viable for at least 96 h.

Following incubation with the 2 highest concentrations of MQ (100 and 75 μg/ml) schistosomula died rapidly; no movement was observed 3–4 min after incubation. At this time-point, schistosomula incubated with 1–10 μg/ml of MQ showed normal movements, while schistosomula incubated with 15–50 μg/ml of MQ showed slowed activity (Fig. 1).

At 1 h after incubation with 25 and 50 μg/ml of MQ all worms had died. Schistosomula incubated with 5, 10 and 15 μg/ml showed only minimal activity, including convulsions and contractions. Severe morphological changes, such as dark granular and crescent-shaped bodies associated with a reduction in body lengths were seen. No effect was observed at the lowest concentration of MQ (1 μg/ml) at the 1 h time-point. Hence, the MLC and MEC of MQ on schistosomula determined after 1 h were 25 and 5 μg/ml, respectively. In the presence of 1 μg/ml of MQ schistosomula revealed first morphological changes and a decreased motility 48 h post-incubation and 96 h after incubation most of the schistosomula were dead.

The structure and appearance of *S. mansoni* schistosomula, incubated for 1 h in the absence of MQ were similar to those described in the literature (Crabtree and Wilson, 1980; Basch and Basch, 1982). In brief, schistosomula had a round shape, clearly defined head and tail regions and an incompletely developed ventral (acetubulum) and oral sucker (Fig. 2A). The tegument was covered by tegumental spines, which were directed towards the posterior end (Fig. 2B).

Thirty min after incubation with 10 and 100 μg/ml of MQ, all schistosomula examined showed a change in shape and extensive tegumental alterations. Worms incubated with 100 μg/ml of MQ showed intensive blebbing and shortened spines on the entire tegumental surface (Fig. 2C). The incubation of schistosomula with 10 μg/ml of MQ showed contraction and bending of the worm body, resulting in the formation of wrinkles and a reduction of body length by $\sim$50%. In addition, spines were shortened (Fig. 2D). Schistosomula exposed to 1 μg/ml of MQ showed no morphological changes 30 min after incubation, and their structure and appearance was similar to the controls.

At 6 h after incubation with 10 μg/ml MQ the tegument of schistosomula showed many deep furrows, swelling and flattened spines (Fig. 2E and F). At this same time-point, the lowest concentration of MQ induced no tegumental alterations on schistosomula.

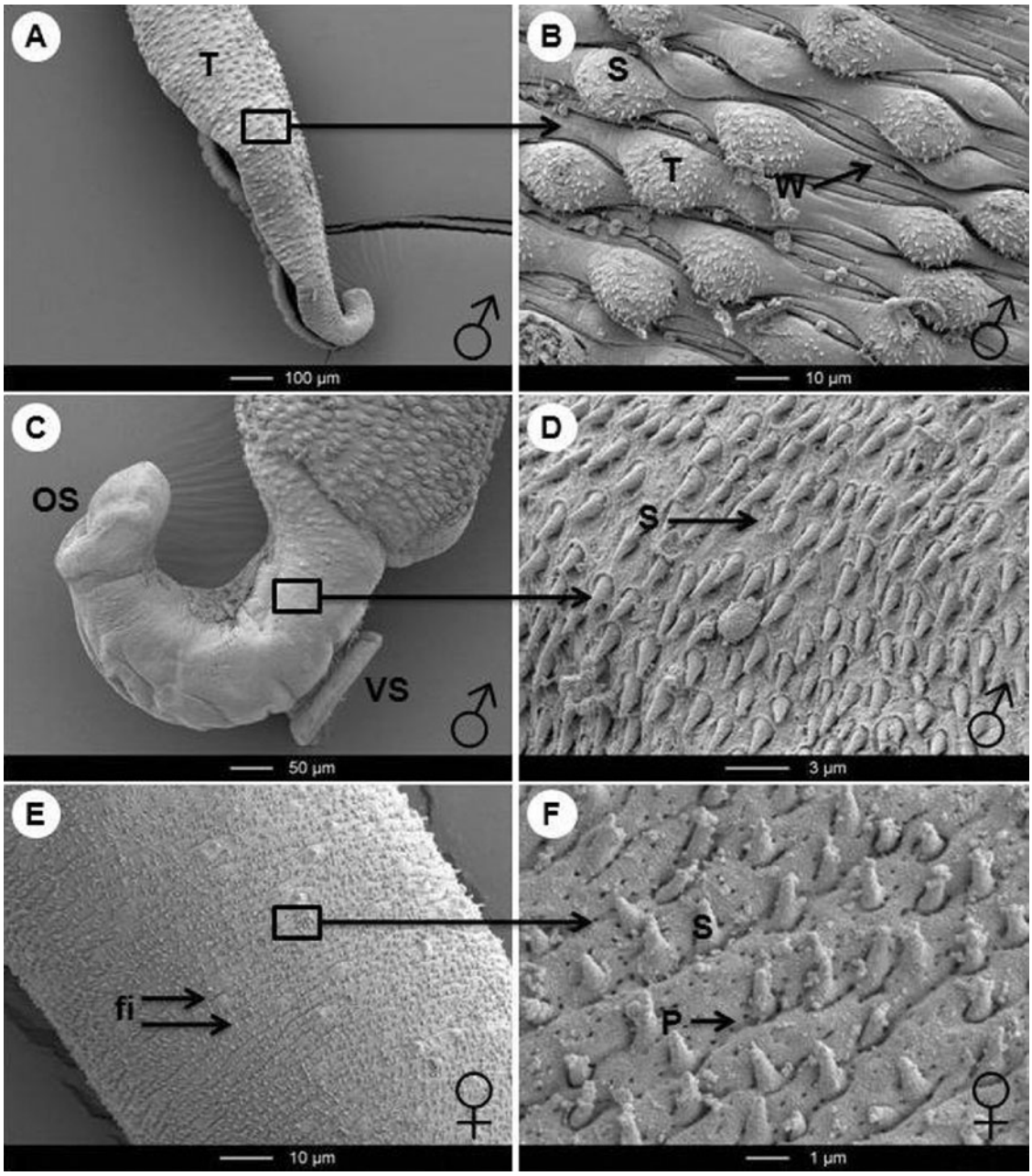

Fig. 4. SEM observations of untreated controls of adult *Schistosoma mansoni* recovered from a mouse 49 days post-infection by portal perfusion. (A) Tail region of male worm, showing tubercles (T). (B) Higher magnification of (A): tegument with tubercles (T), spines (S) and parallel-arranged wrinkles (W). (C) Head region of a male worm, showing the ventral (VS) and the oral sucker (OS). (D) Higher magnification of (C): tegument of the head region with spines (S) visible. (E) Female tegument of the mid-body region with parallel arranged fissures (fi). (F) Higher magnification of (E). Spines (S) and pores (P) visible.

Slight tegumental alterations were observed on worms incubated with 1 μg/ml of MQ after 72 h; schistosomula were bending backwards (Fig. 2G) and showed a shrunken tegument (Fig. 2G and H).

In vitro *studies of adult* S. mansoni – *dissecting microscopic and SEM evaluations*

The dose-response relationships of MQ on adult schistosomes for up to 72 h are given in Fig. 3. Control female and male schistosomes showed normal viability for up to 72 h. Egg production was observed starting 24 h post-incubation. After 3–4 min of incubation with 100 μg/ml of MQ, schistosomes displayed only minimal activities, including slow convulsions and contractions. At 1 h after incubation, adult worms exposed to this concentration were dead (Fig. 3). Worms incubated with 10 and 1 μg/ml of MQ showed normal activity at the 1 h time-point. After 24 h, all schistosomes incubated with 10 μg/ml of MQ were dead. In the presence of 1 μg/ml of MQ, there was a reduction in

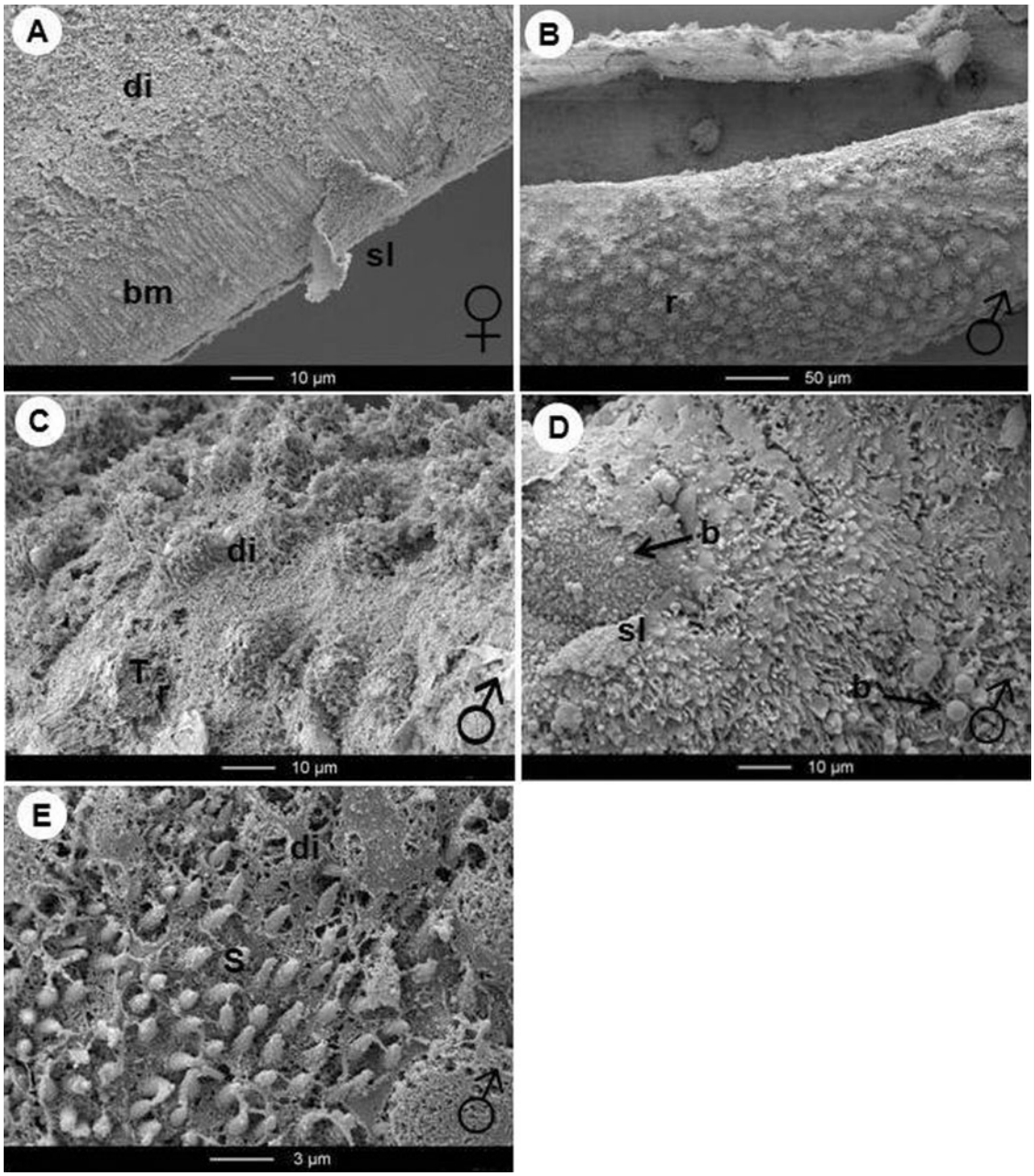

Fig. 5. (Cont.)

the viability of female worms after 72 h compared with untreated schistosomes. MQ suppressed egg production in female worms incubated with all 3 concentrations used.

The tegumental surface of adult *S. mansoni* has been described in a number of SEM studies (Miller *et al.* 1972; Hockley, 1973; Hockley and McLaren, 1973). Briefly, the surface of female schistosomes is simpler and more uniform when compared with the male worm (Basch and Basch, 1982; Gupta and Basch, 1988). The latter sex showed many tubercles on the dorsal body surface of the tail region (Fig. 4A). At a higher magnification, spines on the top of the tubercles and parallel-arranged wrinkles on the tegument were visible (Fig. 4B). The head region of both sexes of *S. mansoni* is characterized by an oral and a ventral sucker (Fig. 4C) and a smooth tegument with numerous tegumental spines (Fig. 4D). The mid-body region of female adults had parallel arranged fissures (Fig. 4E) and, at a higher magnification, tegumental spines and pores were visible (Fig. 4F).

By 30 min after incubation with 100 μg/ml of MQ extensive destruction was apparent on the entire tegument of all adult schistosomes examined. For example, on the mid-body region of female worms, extensive sloughing was visible, leaving the basal membrane exposed. In addition, the tegument showed a roughened appearance and started to disintegrate (Fig. 5A). Figure 5B depicts a roughened surface of a male specimen; at a higher magnification, disintegration of the tegument, resulting in a fibrous

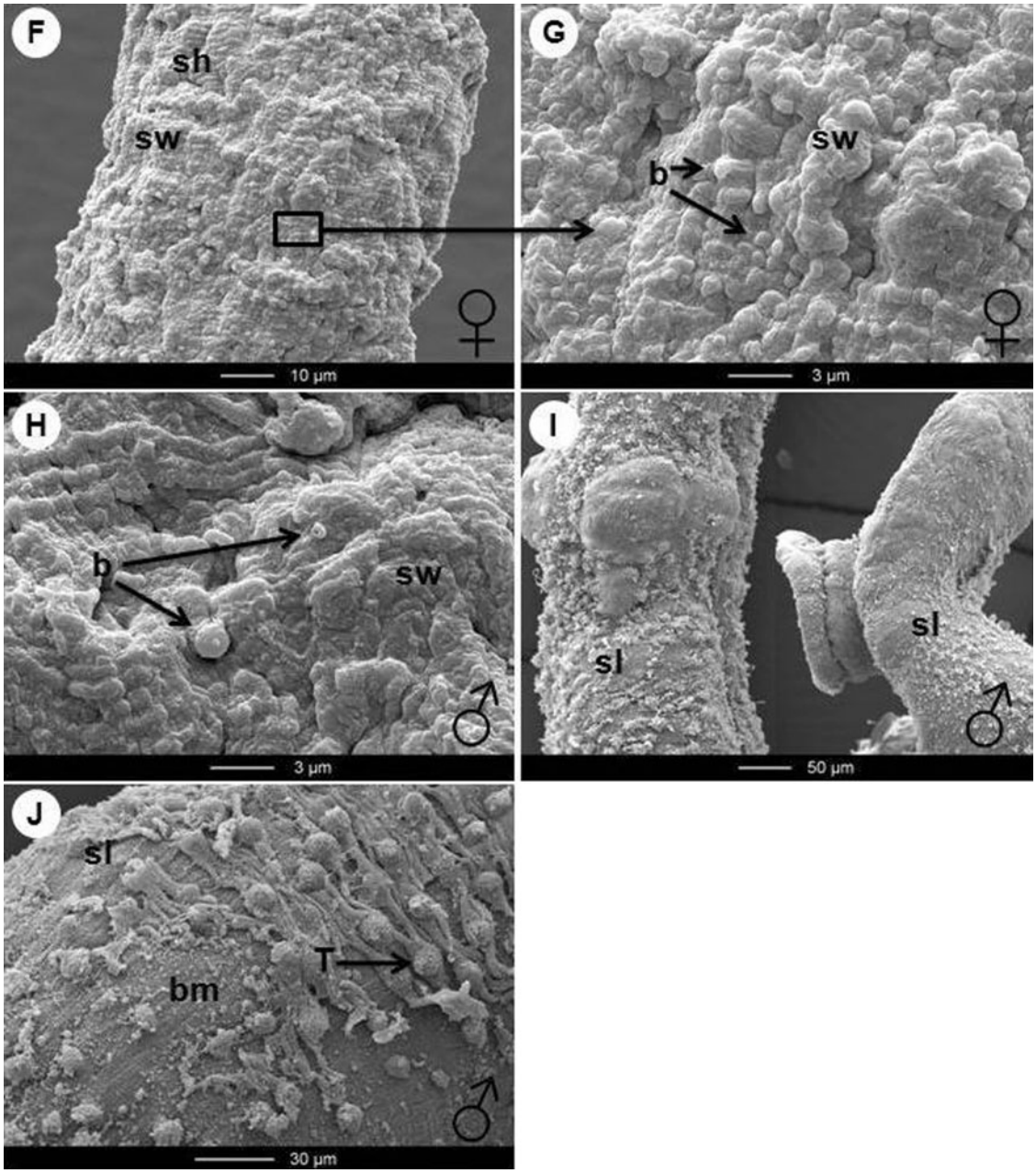

Fig. 5. (Cont.)

appearance, loss of tubercles, spines and the parallel-arranged wrinkles, was visible (Fig. 5C). The whole tegumental layer was destroyed.

After 1 h of incubation with 10 μg/ml of MQ, tegumental alterations were observed on the oral suckers of male worms and on the anterior and mid-body regions of both males and females. For example, blebbing and sloughing were observed on the head region of a male worm. Even the inner surface of the oral sucker of this worm was affected (Fig. 5D). Higher magnification revealed a disintegration of the interspinal tegument of the oral sucker, resulting in a roughened and fibrous appearance, with a loss of regularly arranged spines (Fig. 5E). A roughening of the tegument was also observed on the ventral suckers of male worms (not shown). While part of the female mid-body regions showed no damage, other mid-body regions revealed heavy sloughing, leaving the underlying membrane exposed.

After incubation for 24 h with 10 μg/ml of MQ, massive shrinking and swelling of the tegument was observed on all female worms examined (Fig. 5F). At a higher magnification of the ventral mid-body region, numerous blebs and swelling of the tegument, associated with a loss of spines, fissures and pores were seen (Fig. 5G). Similar observations were made when the tegument of the male flukes was examined. The male tegument was swollen and showed some blebs (Fig. 5H).

By 48 h after incubation with 10 μg/ml of MQ, sloughing affected the whole bodies of male and female worms. The ventral suckers were also affected

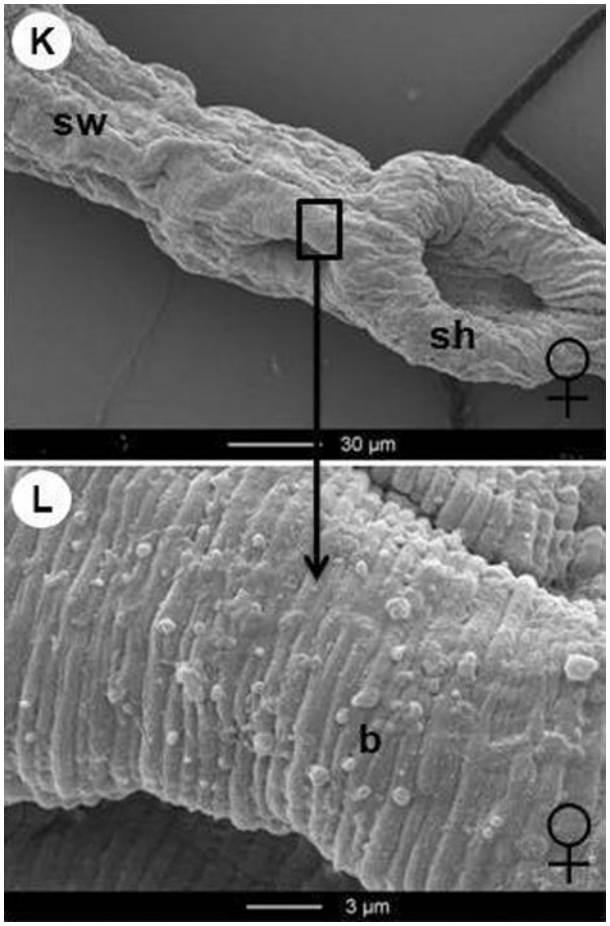

Fig. 5. SEM observations of adult *Schistosoma mansoni* after *in vitro* incubation with MQ. (A) Thirty min after incubation with MQ (100 µg/ml). Tegument of the mid-body region of a female worm. Extensive sloughing (sl) exposing to view the basal membrane (bm). Tegument shows roughening and disintegration (di). (B) Thirty min after incubation with MQ (100 µg/ml). Lateral and dorsal tegument around the gynecophoral canal of a male worm depicting a roughened (r) surface. (C) Higher magnification of (B). Tubercles (T) and the lateral tegument disintegrate (di) resulting in disappearance of the knobs, spines and the parallel-arranged wrinkles. (D) One h after incubation with MQ (10 µg/ml). Blebbing (b) and sloughing (sl) on the inner surface of the oral sucker of a male specimen is visible. (E) Higher magnification of (D) reveals disintegration (di) of the tegument. Spines (S) are still apparent, but not as clear and regular as in the control. (F) Twenty-four h after incubation with MQ (10 µg/ml). The tegument of the ventral mid-body region of a female worm is shrunken (sh) and swollen (sw). (G) Higher magnification of (F) shows many blebs (b) and swelling (sw) of the tegument. (H) Twenty-four h after incubation with MQ (10 µg/ml). Swollen (sw) male tegument of the ventral mid-body region with many blebs (b) seen. (I) Forty-eight h after incubation with MQ (10 µg/ml). Sloughing (sl) on the anterior region and at the ventral sucker of a male worm visible. (J) Forty-eight h after incubation with MQ (10 µg/ml). Mid-body region of a male worm: tegument with tubercles (T). The tegument shows extensive sloughing (sl). In some parts it lifted away leaving the basal membrane (bm) exposed. (K) Forty-eight h after incubation with MQ (1 µg/ml). Female with a shrunken (sh) and swollen (sw) mid-body tegument. (L) At higher magnification of this body region numerous blebs (b) were seen.

(Fig. 5I). In many parts, the tegument lifted away and left the basal membrane exposed. In addition, tubercles had lost their spines (Fig. 5J). Additionally, many blebs of different sizes were visible on the basal membrane. At the same time-point, worms incubated with the lowest concentration of MQ (1 µg/ml) also revealed tegumental alterations with a more severe tegumental response observed on female worms. The female body was shrunken and formed many wrinkles and deep furrows. In addition, the tegument was swollen in some parts and blebbing was observed (Fig. 5K and L). On the other hand, the teguments of male worms were only slightly roughened.

In vivo *studies of adult* S. mansoni – *hepatic translocation*

The distribution of adult worms in the mesenteric veins and the liver of *S. mansoni* infected mice 24, 48, 72, 96 and 120 h post-treatment with MQ (400 mg/kg) is summarized in Table 1. A hepatic shift was already observed 24 h post-treatment with MQ, with 61·8% of *S. mansoni* harboured in the liver. At 72 h and 120 h post-treatment >92% and >98%, respectively, of all worms had shifted to the liver.

In vivo *studies of adult* S. mansoni – *dissecting microscope and SEM evaluations*

All adult flukes recovered from the mesenteric veins of mice at 24, 48, 72, 96 and 120 h after MQ treatment were alive and viable. Worms which had shifted to the liver could not be examined.

Twenty-four h after *S. mansoni*-infected mice had received a single oral dose of MQ (400 mg/kg) localized blebbing on the tegument was the main response to drug action observed on worms recovered from the mesenteric veins (Fig. 6A). By 48 h post-treatment, blebbing increased, affecting the entire tegumental surface of the worms (Fig. 6B).

At 72 h after treatment, tegumental damage was more severe and several worms, in particular the females, were now affected. For example, the mid-body tegument of a female *S. mansoni* became shrunken (Fig. 6C). Again, many blebs were visible on the teguments as depicted in Fig. 6D. The oral sucker of a male specimen had a shrunken appearance resulting in formation of deep furrows. Moreover, some parts of the oral sucker around the pharyngeal opening were swollen. Small spines, which cover the inner surface and the outer margin of the oral sucker were not affected (Fig. 6E).

Table 1. Hepatic shift test following a single 400 mg/kg oral dose of MQ administered to mice infected with *Schistosoma mansoni*

Time-point of analysis (h post-treatment)	No. of mice investigated	No, of worms in mesenteric veins		No. of worms in liver		Total worm burden
		Mean (+/−S.D.*)	%	Mean (+/−S.D.)	%	Mean (+/−S.D.)
Control	6	42·6 (11·7)	97·3	1·2 (1·0)	2·7	43·8 (12·0)
24	4	14·3 (11·2)	37·9	23·3 (11·8)	61·8	37·7 (9·2)
48	4	29·5 (14·2)	56·3	25·5 (7·9)	46·3	55·0 (10·6)
72	4	5·7 (9·8)	7·6	69·0 (13·5)	92·4	74·7 (4·7)
96	4	3·0 (3·8)	6·3	44·8 (10·8)	93·7	47·8 (11·3)
120	3	0·5 (0·5)	1·1	44·0 (9·0)	98·9	44·5 (9·5)

* S.D., standard deviation.

At 96 h after treatment a slightly increased tegumental response to drug treatment was observed on female worms when compared to previous time-points. For example, the mid-body region of a female worm examined showed a shrunken appearance with deep furrows (Fig. 6F).

DISCUSSION

Recently, promising anti-schistosomal properties of MQ against both adult and juvenile schistosomes harboured by mice were found (Keiser *et al*. 2009) and proof-of-concept trials have been launched with MQ in *S. mansoni* or *S. haematobium*-infected children in geographical regions in which malaria and schistosomiasis are co-endemic. To further deepen our understanding of the activity of this anti-malarial drug against schistosomes, we investigated morphological effects and tegumental alterations of MQ on *S. mansoni* juveniles (schistosomula) and adults *in vitro* and *in vivo*. Dissecting microscopic investigations demonstrated the temporal effect of MQ on viability and morphology as well as dose-response relationships on schistosomula and adult *S. mansoni*. In addition, SEM examinations revealed the progressive stages of damage to and disruption of the tegumantal surface of the worm.

MQ had a very fast onset of action on schistosomula *in vitro*. In the presence of 100 and 75 μg/ml worms died immediately. Concentrations of $\geqslant$25 μg/ml killed all schistosomula after 1 h of incubation. Interestingly, a slightly slower onset of action was observed when adult worms were incubated with MQ *in vitro*. For example, while 10 μg/ml of MQ killed schistosomula already after 6 h, the adults did not die until 24 h of exposure *in vitro*. A much slower onset of action was observed on adult worms collected following *in vivo* treatment with MQ. Worms collected from the mesenteric veins were still alive up to 96 h following treatment, and only moderate tegumental disruption, such as blebbing and a shrunken body, was observed. However, severely damaged worms might have already been expelled and worms that had translocated to the liver could not be examined. Hence, the evidence presented might have underestimated the amount of damage inflicted by the drug to the worm population as a whole. Nonetheless, the discrepancy between the effect of the drug and the onset of action of MQ *in vitro* and *in vivo* might be related to lower MQ concentrations present in the livers and mesenteric veins in mice compared with *in vitro* results. Pharmacokinetic (PK) studies, measuring drug concentrations in the body and the target organs, might aid elucidation of these differences observed *in vitro* and *in vivo*. In addition, the effect as well as onset of action of MQ on different stages of juvenile worms (e.g. 21-day-old worms) *in vitro* and *in vivo* remains to be elucidated.

Interestingly, worms incubated with MQ *in vitro* died without the presence of haemin or red blood cells. In contrast, no apparent effect was observed when schistosomes were incubated with the anti-malarial artemether *in vitro* in the absence of haemin, suggesting that artemether interacts with haemin to exert a toxic effect on the worms (Xiao *et al*. 2001). Though the exact mechanism of action of MQ on malaria parasites has not yet been entirely elucidated, a disturbance of the haemoglobin metabolism due to inhibition of the haematin polymerization and formation of an insoluble polymer, termed haemozoin, has been suggested (Dorn *et al*. 1998). A recent morphological study of *S. japonicum* in mice, showed a pronounced dilatation of the gut of adult worms, accompanied by focal or extensive peeling of gut epithelial cells, or even focal collapse of the gut wall (Zhang *et al*. 2009). Erythrocytes with accumulated MQ entering the worm gut might inhibit haem detoxification, resulting in gut damage (Zhang *et al*. 2009). Hence, while an interference of MQ with the haemoglobin digestion of schistosomes might play a role *in vivo*, MQ seems to attack additional drug targets *in vitro*. Further studies are necessary to elucidate the multiple mechanisms of action of MQ, which seems to be involved in the killing of schistosomes. It might also be useful to investigate whether

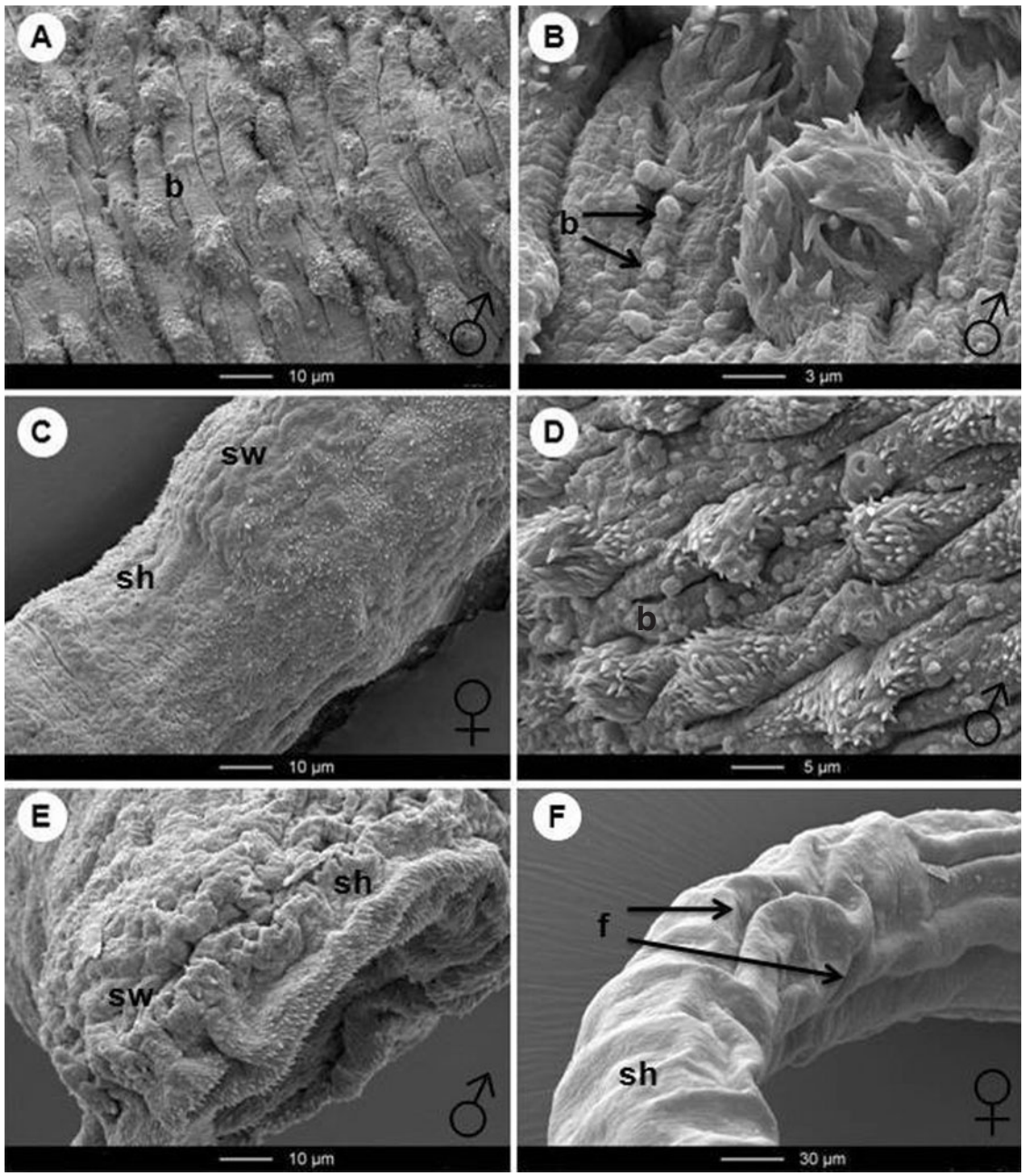

Fig. 6. SEM observations of adult *Schistosoma mansoni* recovered from mice treated with a single oral dose of MQ (400 mg/kg). (A) Twenty-four h after treatment. Blebbing (b) on the dorsal mid-body tegument of a male worm is visible. (B) Forty-eight h after treatment. Male dorsal tegument showing blebs (b). (C) Seventy-two h after treatment. Tegument of the mid-body region of a female has a shrunken (sh) and swollen (sw) appearance. (D) Seventy-two h after treatment. Dorsal mid-body male tegument reveals many blebs (b). (E) Seventy-two h after treatment. Oral sucker (OS) of a male specimen shows a shrunken (sh) and swollen (sw) tegument. (F) Ninety-six h after treatment. The shrunken (sh) mid-body region of a female *S. mansoni* with many furrows (f) is visible.

MQ acts synergistically with the host immune response, similar to the chemotherapeutic effect of PZQ, which has been shown to be dependent on the host antibody response (Brindley and Sher, 1987).

SEM is often used for documenting the efficacy of anti-schistosomal drugs. The 10–20 μm thick tegument (Neves *et al*. 2001) that covers the entire surface of schistosomes represents an important drug target. It consists of a double outer membrane (2 lipid bilayers), has no glycocalyx (Hockley, 1973; Wilson and Barnes, 1974; McLaren and Hockley, 1977), is responsible for absorptive activities (Rogers *et al*. 1983) and is a protection barrier between the parasite and the host (Hoffmann and Strand, 1996). In addition, it contains numerous ciliated or unciliated bulb-shaped tubercles, which are sensory receptors and associated with ring-shaped actin filaments (Zhou and Podesta, 1992). Also, the spines of the tubercles contain bundles of highly packed actin filaments (Zhou and Podesta, 1992).

We observed extensive tegumental damage on the larvae and adult developmental stages of *S. mansoni*, in particular following *in vitro* incubation, which intensified progressively as the incubation period and the concentration of MQ increased. Blebbing was visible on the tegument of both treated juvenile and adult flukes. Blebbing is an indicator for stress and has been observed in previous SEM studies evaluating anti-schistosomal drugs (Jiraungkoorskul *et al*. 2005). Nonetheless, vesicle formation induced by MQ might also occur due to the focal lysis of muscles (Zhang *et al*. 2009). Interestingly, while in the present study blebbing was observed on the tegument of both male and female adult *S. mansoni*, vesiculation was absent from female *S. japonicum* (Zhang *et al*. 2009). Flattened spines were observed on the tegument of schistosomula and roughening, sloughing and disintegration of the tegument were other typical features observed on the tegument of adult worms in the present investigation. In addition, extensive swelling and furrowing were observed on both development stages.

The effect of PZQ on the tegument of *S. mansoni* shows distinct differences, when compared to MQ, though related tegumental alterations such as sloughing, swelling, and loss or shortening of spines have also been documented for PZQ (Xiao *et al*. 2000). First, in contrast to small- and also medium-sized blebs observed in the present study a typical feature seen on the tegument of *S. mansoni* treated with PZQ was the presence of large-sized vesicles protruding from the tegumental surface, most likely due to swelling of the cytoplasm (Xiao *et al*. 1981). Second, in the current study, the tegument of female worms was slightly more affected and females died more rapidly when compared to male worms, in particular following *in vitro* exposure to MQ, male worms had a higher sensitivity to PZQ than female worms *in vitro* (Pica-Mattoccia and Cioli, 2004) and exhibited more extensive tegumental damage than female worms following PZQ treatment (Shaw, 1990). The greater MQ susceptibility of females is consistent with results from a previous *in vivo* study (Keiser *et al*. 2009), in which greater reductions in the female worm burden were observed compared with total worm burden reductions. We speculated that either a sex-specific interference of the drug with the target might occur or that there are different targets for MQ in females compared with males (Keiser *et al*. 2009). Alterations induced by artemether on the tegument of *S. mansoni* have also been well studied. Briefly, severe tegumental damage was observed on juvenile schistosomes, correlating with the high efficacy of artemether in killing 21-day-old schistosomes (Xiao *et al*. 2000). Focal swelling and fusion of tegumental ridges and occasionally sloughing was observed on adult *S. mansoni* recovered from mice. Similar to the present results obtained using MQ, female worms were more affected following treatment with artemether (Xiao *et al*. 2000).

We documented distinct morphological characteristics of schistosomes incubated with MQ. Schistosomula treated with MQ had a crescent-shaped body and showed contraction resulting in a reduction of the body length by approximately half. In addition, a dark granular body was seen. Adult worms showed convulsions after incubation with MQ *in vitro*. In contrast, rapid spasmodic contractions of the worm body are the most prominent morphological alterations induced by PZQ (Xiao *et al*. 1984). Finally, *in vivo* studies showed that extensive structural changes to worms occurred within 15 min of treatment, and both female and male adult worms died within 24 h following treatment with PZQ (Shaw, 1983; Xiao *et al*. 1983). In contrast, in our study worms were still alive several days post-treatment of mice with MQ and nearly all worms had shifted to the liver after 72 h. Interestingly, a more rapid hepatic translocation was observed with MQ on *S. japonicum*, all worms had moved to the liver 48 h after treatment (Zhang *et al*. 2009).

In conclusion, we demonstrated that MQ induces extensive morphological and tegumental alterations on both *S. mansoni* schistosomula and adults *in vitro* and *in vivo*. Importantly, given the distinct morphological and tegumental changes caused by MQ compared with PZQ, the only other effective anti-schistosomal drug available, we speculate that both drugs attack different targets; hence, it is suggested that no cross-resistance will occur. Thus, the present findings provide a sound basis for further in-depth studies of the anti-schistosomal properties of MQ, both in the laboratory and in the field.

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