In vitro and *in vivo* trematode models for chemotherapeutic studies

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SUMMARY

Schistosomiasis and food-borne trematodiases are chronic parasitic diseases affecting millions of people mostly in the developing world. Additional drugs should be developed as only few drugs are available for treatment and drug resistance might emerge. *In vitro* and *in vivo* whole parasite screens represent essential components of the trematodicidal drug discovery cascade. This review describes the current state-of-the-art of *in vitro* and *in vivo* screening systems of the blood fluke *Schistosoma mansoni*, the liver fluke *Fasciola hepatica* and the intestinal fluke *Echinostoma caproni*. Examples of *in vitro* and *in vivo* evaluation of compounds for activity are presented. To boost the discovery pipeline for these diseases there is a need to develop validated, robust high-throughput *in vitro* systems with simple readouts.

Key words: *Schistosoma mansoni*, *Fasciola hepatica*, *Echinostoma caproni*, *in vitro*, *in vivo*, drug discovery, chemotherapy.

INTRODUCTION

Thus far approximately 6000 species in the sub-class Digenea, phylum Platyhelminthes have been described in the literature. Among them, only a dozen or so species parasitize humans. These include the blood flukes (five species of *Schistosoma*), liver flukes (*Clonorchis sinensis*, *Fasciola gigantica*, *Fasciola hepatica*, *Opisthorchis felineus* and *Opisthorchis viverrini*), lung flukes (*Paragonimus* spp.) and intestinal flukes (e.g. *Echinostoma* spp. and *Fasciolopsis buski*) (Cox, 1993; Keiser and Utzinger, 2009). These parasites are the causative agents of a complex of some acute, but mainly chronic infections: schistosomiasis and food-borne trematodiases (Keiser and Utzinger, 2007; Utzinger *et al*. 2007; Davis, 2009; Sithiathaworn *et al*. 2009). It is currently estimated that 1400 million people are at risk of schistosomiasis and food-borne trematodiases with more than 250 million infections (Keiser and Utzinger, 2005; Steinmann *et al*. 2006). Whilst the global burden of schistosomiasis has been estimated at 1·7–4·5 million disability-adjusted life years (Utzinger and Keiser, 2004; Hotez *et al*. 2009), that of food-borne trematodiases remains to be investigated (Keiser and Utzinger, 2009). Schistosomiasis and food-borne trematodiases belong to the so-called neglected tropical diseases (Hotez *et al*. 2009; Keiser and Utzinger, 2009). The global strategy for the control of schistosomiasis and food-borne trematodiases and other parasitic worm infections is morbidity control by chemotherapy. However, only two drugs are currently available: triclabendazole against fascioliasis and praziquantel against the other food-borne trematode infections and schistosomiasis (Keiser and Utzinger, 2004; Keiser *et al*. 2005). Hence, there is a need for discovery and development of new drugs, particularly in view of growing concern about resistance developing to existing drugs. Triclabendazole resistance is already common in veterinary parasitology (Keiser *et al*. 2005) and the extensive use of praziquantel resistance in mass drug administration programs has raised concern regarding the selection of drug resistant schistosomes (Melmann *et al*. 2009).

There has been little incentive to invest in the discovery and development of trematodicidal drugs. While public-private partnerships have been formed for some of the neglected tropical diseases (e.g. the Drugs for Neglected Diseases Initiative (DNDi) focusing on human African trypanosomiasis and leishmaniasis), devoted drug discovery and development programmes do not yet exist for any of the major helminth diseases (Ridley and Kita, 2007). Moreover, funding for basic research on these parasites and the pathogenesis of neglected tropical diseases has been inadequate (Renslo and McKerrow, 2006). To date, *in vitro* cultivation of trematodes lags behind the cultivation of protozoa or bacteria and cultivation throughout the entire life cycle is not possible (Smyth and Halton, 1983). Additionally, only very few digeneans are maintained in the laboratory, primarily due to their complex life cycles. Life cycles requiring at least 2 hosts, a vertebrate and a molluscan host, are the rule among digeneans

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(Cox, 1993; Gryseels *et al*. 2006; Keiser and Utzinger, 2009). Finally, though target-based trematodicidal drug discovery is starting to bear fruit (Caffrey, 2007; Sayed *et al*. 2008; Abdulla *et al*. 2009), translation of targets identified through comparative genome searches and microarray analysis into validated targets will require substantial economic efforts (Renslo and McKerrow, 2006).

In the present article, I review *in vitro* and *in vivo* trematode models available for chemotherapeutic studies. Emphasis is placed on screening against whole parasites since a molecular-target approach is still rarely employed in trematodicidal drug discovery. The focus is on a blood fluke (*Schistosoma mansoni*), a liver fluke (*Fasciola hepatica*) and an intestinal fluke (*Echinostoma caproni*). While *S. mansoni* and *F. hepatica* are major human pathogens, *E. caproni* is a rodent and avian fluke, which is widely and effectively used as a laboratory model. Examples of *in vitro* and *in vivo* evaluation of compounds for activity against these three selected flukes are given. I conclude that for better targeted future trematodicidal drug discovery there is a strong need to develop validated, robust high-throughput *in vitro* systems with simple readouts.

SCHISTOSOMA MANSONI

S. mansoni *is an important model to study schistosomiasis*

S. mansoni is the most widely used schistosome model, and its life cycle is kept in many institutions worldwide. Fig. 1 summarizes laboratories in Europe, USA, South America, Asia and Northern Africa, which currently maintain the life cycle of *S. mansoni*. Several of these laboratories such as the Schistosomiasis Resource Center (Biomedical Research Institute; Bethesda, USA) or the Theodor Bilharz Research Institute, Giza, Egypt also provide schistosome material for research or teaching purposes. Remarkably, our search did not identify any laboratories in sub-Saharan Africa (the area considered to be most severely affected by schistosome-induced morbidity (van der Werf *et al*. 2003), which maintain the complete laboratory life-cycle of *S. mansoni*.

Briefly, in the definitive host *S. mansoni* worms develop from a post-penetration larval stage, the schistosomulum. The female worms produce eggs which leave the body in excreta – faeces in the case of *S. mansoni*, and on contact of the eggs with fresh water free-swimming miracidia hatch out and infect snails. For laboratory culture miracidia are hatched from *S. mansoni* eggs taken from experimental animal livers and/or intestines and *Biomphalaria glabrata* is the intermediate host snail species most commonly used (Fig. 2). Following asexual reproduction in the snails, cercariae are shed from the snails into freshwater (Lewis, 2001).

S. haematobium is much less studied, as it is difficult to grow in rodents (Doenhoff *et al*. 2009). To my knowledge, the life cycle of *S. haematobium* is currently maintained only in two laboratories, namely the Theodor Bilharz Research Institute (Giza, Egypt) and the Schistosomiasis Resource Center. 'Sibling' species of *S. haematobium*, such as *S. bovis*, *S. matthei* or *S. margrebowiei*, the life cycles of which can be maintained in laboratory rodents, might serve as models for *S. haematobium* (Agnew *et al*. 1989).

S. japonicum, the third major human schistosome species, is mainly studied in China. Nonetheless several institutions outside China maintain the life cycle and infected snails and mammals are also available on request from the Schistosomiasis Resource Center. Though less frequently used, schistosome species infecting other species of definitive hosts might also prove useful for experimental studies: for example, the bird schistosome *Trichobilharzia ocellata* reaches oviposition after 9 days already and is not pathogenic. Its life cycle has been described as troublesome (Smyth and Halton, 1983).

S. mansoni in vitro *assay based on schistosomula*

Following the establishment of the *S. mansoni* life cycle in the laboratory, *in vitro* parasite culture techniques were developed (Ramirez *et al*. 2007). Early work with schistosomula was based on the recovery of young worms from the lungs of infected rodents, hence only limited worm material was available (Smyth and Halton, 1983). The discovery that schistosomula can be obtained by transforming cercariae using simple techniques as centrifugation, repeated aspiration through a syringe needle or chemical stimulation was a breakthrough in the field of schistosome cultivation. Today cercariae are often used as starting material for schistosome *in vitro* studies as large numbers of schistosomula can be obtained in a cost-effective manner (Table 1, Fig. 2) (Smyth and Halton, 1983; Abdulla *et al*. 2009; Caffrey *et al*. 2009). In addition, most importantly, the use of mechanically obtained schistosomula reduces and replaces the use of live animals in accordance with the 3Rs (reduce, replace, refine) of animal protection principles (Broadhead and Bottrill, 1997). An assay with artificially produced schistosomula might serve as a pre-screen in drug sensitivity assays, however one has to keep in mind that mechanically transformed schistosomula possess distinct differences to schistosomula, which have penetrated the skin (e.g. biochemical differences) or juvenile and adult schistosomes (Brink *et al*. 1977).

Several techniques for transforming cercariae and maintaining schistosomula are available (Colley and Wikel, 1974; Howells *et al*. 1974; Ramalho-Pinto *et al*. 1974; Brink *et al*. 1977; Smyth and Halton,

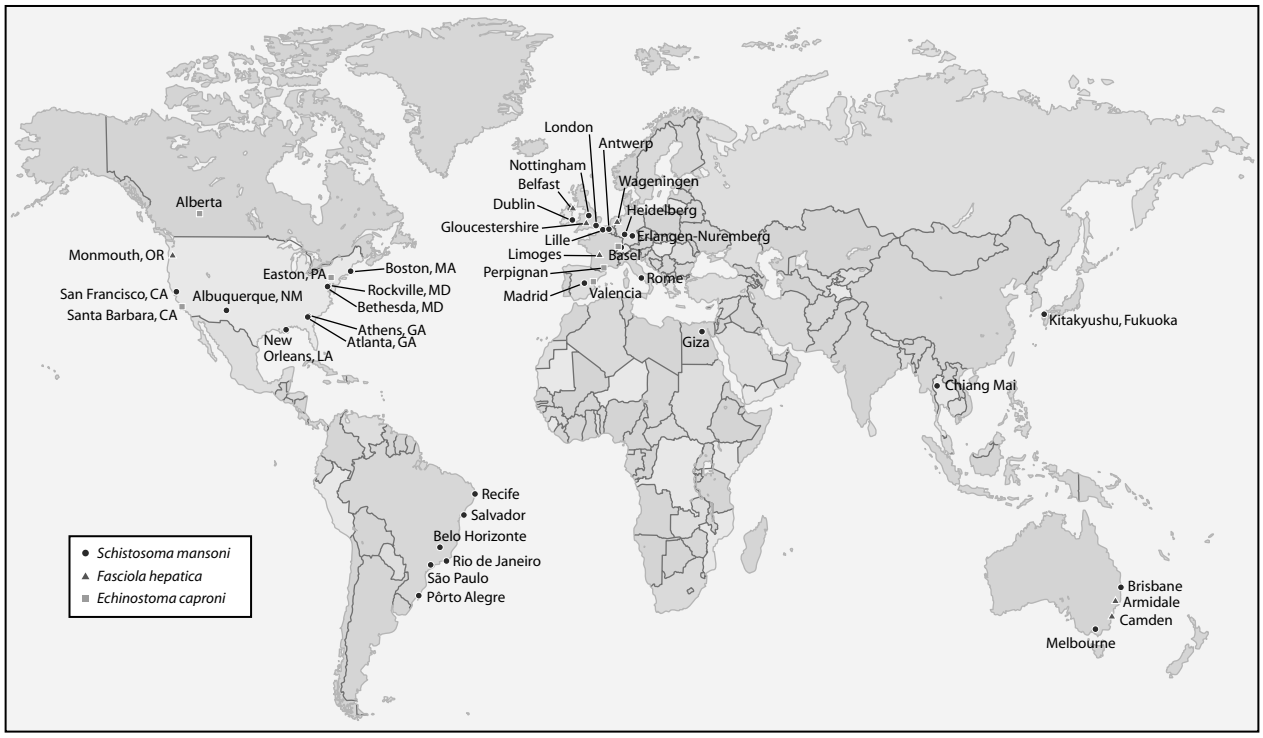

Fig. 1. World map highlighting locations of laboratories, which maintain the life cycles of *S. mansoni* (circles), *F. hepatica* (triangles) and *E. caproni* (squares). Laboratories were identified through contacting experts and literature searches (using PubMed) restricted to the past 5 years.
Schistosoma mansoni: Aggeu Magalhães Institute (FIOCRUZ) (Recife, Brazil), Biomedical Research Institute (Rockville, MD, USA), Butantã Institute, São Paulo (São Paolo, Brazil), Catholic University of Rio Grande do Sul (Porto Alegre, Brazil), CDC Atlanta (Atlanta, GA, USA), Chiang Mai University (Chiang Mai, Thailand), DBL-Centre for Health Research and Development (Copenhagen, Denmark), Federal University of Rio de Janeiro (Rio de Janeiro, Brazil), GIDE – Federal University of Minas Gerais (Belo Horizonte, Brazil), Gonçalo Muniz Institute (FIOCRUZ) (Salvador, Brazil), Institut Pasteur de Lille (Lille, France), Instituto René Rachou-Fiocruz (Belo Horizonte, Brazil), London School of Hygiene and Tropical Medicine (London, UK), Natural History Museum (London, UK), National Research Council (Rome, Italy), Oswaldo Cruz Institute (FIOCRUZ) (Rio de Janeiro, Brazil), Queensland Institute of Medical Research (Brisbane, Australia), Swiss Tropical Institute (Basel, Switzerland), Theodor Bilharz Research Institute (Giza, Egypt), Trinity College (Dublin, Ireland), Tufts University (Boston, MA, USA), Tulane University (New Orleans, LA. USA), Universidad San Pablo-CEU (Madrid, Spain), University Erlangen-Nuremberg (Erlangen-Nuremberg, Germany), University New Mexico (Albuquerque, NM, USA), University of Antwerp (Antwerp, Belgium), University of California San Francisco (San Francisco, USA), University of Georgia (Athens, GA, USA), University of Heidelberg (Heidelberg, Germany), University of Melbourne (Melbourne, Australia), University of Nottingham (Nottingham, UK), University of Occupational and Environmental Health (Kitakyushu, Fukuoka, Japan), University of São Paulo (São Paolo, Brazil), University of São Paulo, Ribeirão Preto (São Paolo, Brazil), University of Texas Health Science Center (San Antonio, TX, USA), University of the Health Sciences (Bethesda, MD, USA), University of York (York, UK). ***Fasciola hepatica***: Baldwin Aquatics (Monmouth, OR, USA), Elizabeth Macarthur Agricultural Institute (Camden, Australia), Facultés de Médecine et de Pharmacie (Limoges, France), Queens University of Belfast (Belfast, Northern Ireland), Ridgeway Research Ltd (Gloucestershire, UK), Veterinary Health Research (Armidale, NSW, Australia), Wageningen University (Wageningen, Netherlands). ***Echinostoma caproni***: Lafayette College (Easton, PA, USA), Swiss Tropical Institute (Basel, Switzerland), Universidad de Valencia (Valencia, Spain), Université de Perpignan (Perpignan, France), University of Alberta (Alberta, Canada), University of California Santa Barbara (Santa Barbara, CA, USA).

1983; Abdulla *et al*. 2009; Caffrey *et al*. 2009). In our laboratories, for example, a Vortex transformation method based on Ramalho-Pinto (Ramalho-Pinto *et al*. 1974) is used. Briefly, 50 ml of an ice-cold cercarial suspension is centrifuged and the packed cercariae are resuspended and vortexed for 2 min to trigger tail loss. For the isolation of cercarial bodies from tails, an ice-cold Hank's basal salt solution (HBSS) is added to the cercarial suspension and the tail-rich supernatant is decanted (Manneck *et al*. 2009). Schistosomula are incubated in Basch medium (Basch, 1981) supplemented with serum and antibiotics or other culture media (Smyth and Halton, 1983). Our own studies have shown that schistosomula survive for at least 96 h in different media (Basch, MEM, DMEM or TC 199) regardless of serum supplementation (author's unpublished observations). Abdulla and colleagues have recently

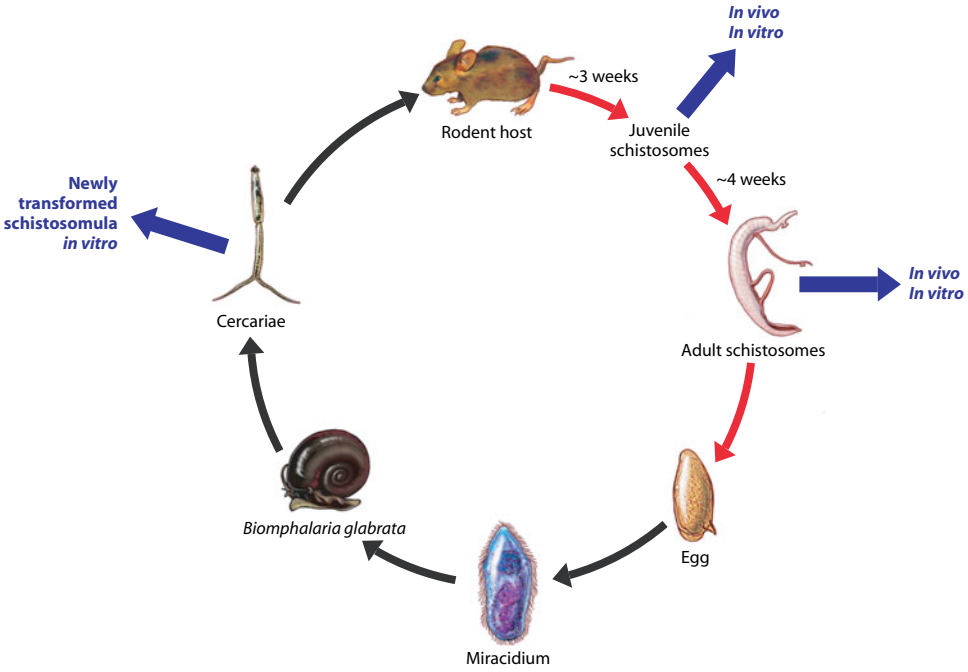

Fig. 2. Life cycle of *S. mansoni* highlighting collection points for *in vitro* and *in vivo* chemotherapeutic studies. Maturation of parasite occurs within final host (red arrows).

reported that schistosomula survive in Basch medium for several weeks (Abdulla *et al.* 2009). In Fig. 3a schistosomula incubated for 24 h in Basch medium are shown. Fig. 3b depicts a scanning electron microscopic (SEM) image of a newly transformed schistosomulum obtained by Vortex transformation. Following incubation of schistosomula with investigational drugs, changes in worm motor activity, morphological/tegumental alterations and occurrence of death are recorded at different time points. These phenotypic changes might be scored using a viability scale from 0 to 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) (Ramirez *et al.* 2007). For example, phenotypic changes following praziquantel incubation (100 μg/ml) for 72 h can be observed by means of light microscopy (Fig. 3c) and SEM (Fig. 3d). Blebbing, furrows, swelling, loss of structure and the lack of spines were visible following incubation with praziquantel. Since the assessment of morphological and behavioural responses of schistosomula to treatment is quite labour intensive and time consuming, the use of a drug response predictor (e.g. colourimetric assays, such as MTT formazan (3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium formazan), which measures the metabolic activity as a marker of viability in mammalian cell culture systems) should be explored. The latter assay technology might offer distinct advantages such as standardization, reproducibility and accuracy and can be performed more rapidly and easily, when compared to phenotypic evaluated drug screens.

Adult S. mansoni in vitro *assay*

In parallel to the development of schistosomula culture systems, culture methods for adult worms have been established (Clegg, 1965; Ramirez *et al.* 2007). Today, *in vitro* chemotherapeutic studies mainly rely on *S. mansoni* adults (Table 1, Fig. 2). For example, standard operating procedures (SOPs) for compound screening of the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) are based on adult worms. Since the adult worm supply is somewhat restricted, the *in vitro* throughput capacity of this assay is limited (~2,000 compounds are screened each year at TDR screening centers) (Ramirez *et al.* 2007). For this assay, mice are infected with a high number (up to 600) of *S. mansoni* cercariae. Adult *S. mansoni* are obtained by portal perfusion of mice after 40 days post-infection (Yolles *et al.* 1947; Smithers and Terry, 1965). 3–4 week old worms might also be used for *in vitro* studies (Hobbs *et al.* 1993) including drug sensitivity assays, as juvenile worms might show differences with regard to drug sensitivity when compared to larvae and adult worms. Following several washing steps, worms are incubated for example in DMEM, RPMI 1640 or HBSS supplemented with foetal calf serum, penicillin and streptomycin (Ramirez *et al.* 2007; Xiao *et al.* 2007). Schistosomes survive in a range of

Table 1. *In vitro* drug screening: techniques employed, culture conditions and evaluation of juvenile and adult *S. mansoni*, *F. hepatica* and *E. caproni* assays

Trematode	*In vitro* assays	Techniques employed	Culture media	Key issues	Evaluation of assay
Schistosoma mansoni	Newly transformed schistosomula	Mechanical or chemical transformation of cercariae	Different media possible, e.g. Bash, MEM, DMEM, TC 199 supplemented with penicillin and streptomycin (and foetal calf serum)	Cost-effective and ethical as large numbers of schistosomula can be obtained and no animals needed. Differences in drug sensitivity between schistosomula and adult worms?	Microscopic assessment of motility (viability score), morphology and death of worms. SEM and TEM studies.
	Schistosomula (juvenile worms)	Juvenile worms collected from lungs of highly infected mice (infected with 400–600 cercariae)	Different media possible, e.g.DMEM, RPMI 1640 or Hanks' balanced salt solution supplemented with foetal calf serum, penicillin and streptomycin	Rarely used in chemotherapeutic studies.	
	Juvenile schistosomes (3–4 week old) Adult schistosomes	Perfusion of the portal venous system of highly infected mice (infected with 400–600 cercariae)		Need of animals. Drug sensitivity assessed on target parasite.	Microscopic assessment of motility (viability score), morphology and death of worms, inability to reduce the tetrazolium, MTT. SEM and TEM studies.
Fasciola hepatica	Newly excysted juveniles	Excystation of metacercariae	RPMI 1640 supplemented with antibiotics (and serum or red blood cells)	Cost-effective and ethical as large numbers of newly excysted juveniles can be obtained and no animals needed. Differences in drug sensitivity between newly excysted juveniles and adult worms demonstrated.	Microscopic assessment of motility, morphology and death of worms, inability to reduce the tetrazolium, MTT. SEM and TEM studies.
	Juvenile worms	Collection of worms from rat or sheep livers	NCTC 135 or RPMI 1640 supplemented with antibiotics (and serum or red blood cells)	Need of animals. Drug sensitivity assessed on target parasite.	Microscopic assessment of motility (viability score), morphology and death of worms. SEM and TEM studies.
	Adult worms	Collection of worms from the bile ducts of rats or sheep	NCTC 135 or RPMI 1640 supplemented with antibiotics (and serum or red blood cells)	Need of animals. Drug sensitivity assessed on target parasite.	
Echinostoma caproni	Juvenile worms	Excystation of metacercariae	NCTC 135 supplemented with antibiotics and serum or egg yolk	Cost effective and ethical as large numbers of newly excysted juveniles can be obtained and no animals needed.	Microscopic assessment of motility (viability score), morphology and death of worms. SEM and TEM studies.
	Adult echinostomes	Collection of worms from the intestine of highly infected mice (up to 300 metacercariae)	RPMI 1640 supplemented with antibiotics and glucose	Need of animals.	

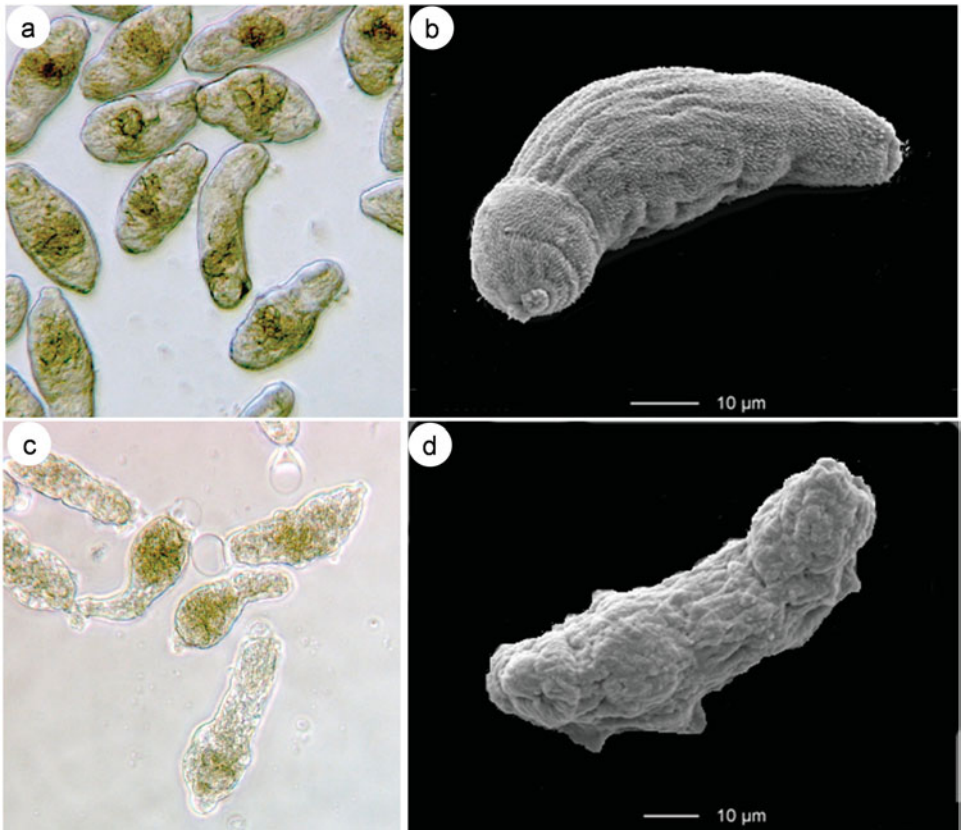

Fig. 3. *Schistosoma mansoni* – (a): light microscopic (LM) image of newly transformed control schistosomula. (b) SEM image of newly transformed control schistosomula. (c) LM image of newly transformed schistosomula incubated for 72 h in the presence of 100 μg/ml praziquantel (d) SEM image of newly transformed schistosomula incubated for 72 h in the presence of 100 μg/ml praziquantel.

supplemented tissue culture media for periods ranging from 3–120 days (Smyth and Halton, 1983). Test drugs at various concentrations (5 μg/ml is the standard concentration in the primary screen at WHO-TDR screening centers (Ramirez *et al*. 2007)) are added to each culture well containing 6–10 worms. Worms are examined under a dissecting microscope at different time points (e.g. 24, 48 and 72 h). Control schistosomes are incubated in the presence of the highest concentration of solvent used. Drug effects are commonly evidenced by phenotypic changes (motility and morphological changes and death) using an inverted microscope as described above. Markers such as MTT formazan have also been used to evaluate the viability of adult schistosomes (Oliveira *et al*. 2004). For more thorough studies on the effects of antischistosomal drugs, detailed surface features of treated and untreated specimens might be investigated by means of SEM as the tegument of trematodes is a highly susceptible site of damage following drug treatment (Manneck *et al*. 2009). For example, in Fig. 4b the male tegument is depicted. Fig. 4c shows the tegument of a male *S. mansoni* following drug treatment: 72 h post incubation with 100 μg/ml praziquantel blebbing was observed.

S. mansoni in vivo *model*

The mouse is the most commonly used mammalian host for experimental studies with *S. mansoni* (Table 2, Fig. 2). Though a difference has been observed regarding the percentage of cercariae that mature in different mouse strains, most mice strains tested were susceptible to an infection with *S. mansoni* (Lewis, 2001). 129/Ola and WEHI 129J mice are two examples of non-permissive hosts (Elsaghier *et al*. 1989). Hamsters (Xiao, *et al*. 2007), gerbils (Chisty *et al*. 2002), rabbits (Kasilima *et al*. 2004) or primates (Webbe *et al*. 1981) have also been used to study *S. mansoni in vivo*. The rat is only a semi-permissive host (Cioli *et al*. 1977) and therefore unlikely to be suitable for the present purpose.

Animals are infected either by skin penetration, usually through the tail or shaved abdominal skin or via subcutaneous or intraperitonal injection of cercariae (Lewis, 2001). For chemotherapeutic studies it is important to compare the number of schistosomes in treated and untreated control animals, which is usually done after schistosomes have matured (6 to 8 weeks after infection).

Schistosomes are obtained and counted either via perfusion of the portal venous system (Yolles *et al*.

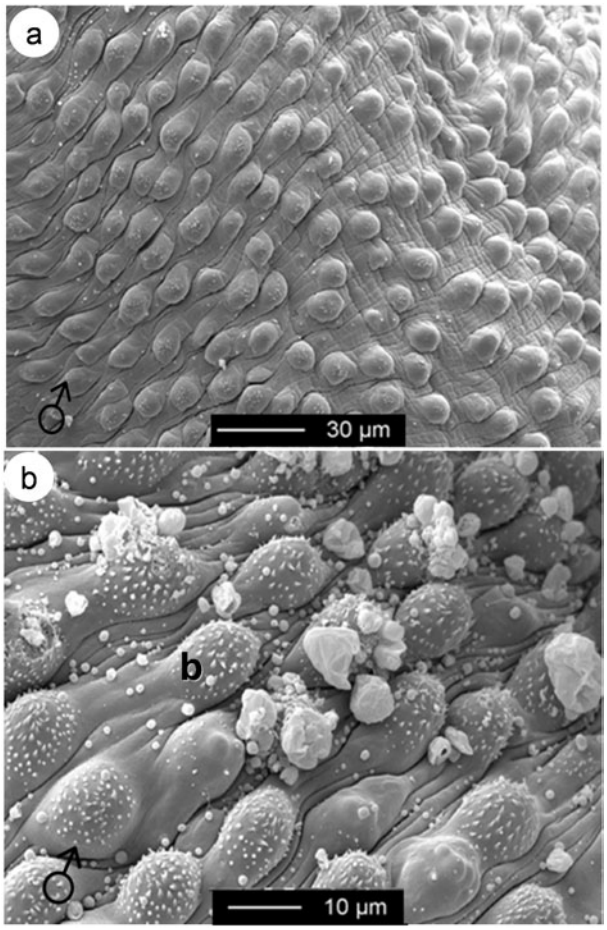

Fig. 4. *Schistosoma mansoni* – SEM observations. (a) dorsal male tegument and (b) blebbing (b) observed on the dorsal male tegument following incubation with 100 µg/ml praziquantel for 72 h.

1947; Lewis, 2001) or examining the mesenteric veins and the liver for the presence of schistosomes (Xiao *et al*. 2007). In the latter case, the liver and the small and large intestines are removed from the animal. The liver is placed into a 20 × 20 cm transparent plastic folder, compressed between 2 glass plates and the parenchyma is examined under a stereoscopic microscope. In addition, the mesenteric veins are systematically examined using a stereoscopic microscope. All schisosomes present in the liver and mesenteric veins are sexed and counted (Xiao *et al*. 2007).

To revisit our example of praziquantel, the current drug of choice, the *in vivo* antischistosomal properties of this drug have been well studied in the *S. mansoni*-mouse model. For example, a single 300 mg/kg oral dose of praziquantel resulted in a worm burden reduction of 81% (Xiao *et al*. 1999).

FASCIOLA HEPATICA

Fascioliasis is a major public health problem and an economically serious disease of livestock. Novel treatment options are of high priority as resistance to triclabendazole, the drug of choice in human and animal medicine, is spreading (Keiser *et al*. 2005; Fairweather, 2009). In addition, triclabendazole is licensed in only 4 countries (Keiser, *et al*. 2005). Chemotherapeutic studies have been undertaken with both *F. hepatica* and *F. gigantica*, although the former species has been more extensively studied.

The life cycle of *Fasciola* spp. is maintained at several research laboratories, universities and private companies (Fig. 1), where metacercariae (different triclabendazole-resistant and -sensitive isolates) can be obtained or purchased. Briefly, the lymnaeid snails *Galba* spp. and *Fossaria* spp. mainly act as intermediate hosts (Mas-Coma *et al*. 2007). Infection in humans and animals occur through the ingestion of metacercariae attached to water vegetables or in contaminated water (Keiser *et al*. 2005; Mas-Coma *et al*. 2007; Keiser and Utzinger, 2009). In experimental infections laboratory animals are exposed to metacercariae by stomach tube or oesophageal intubation, while a gelatin bolus or capsule containing the metacercariae are often used to infect sheep (Clery *et al*. 1995). In the intestine the fluke excysts (newly excysted juvenile), penetrates the gut wall and enters the body cavity. After wandering over the viscera the juvenile flukes reach the liver. For several weeks the juvenile flukes burrow through the liver causing hemorrhage, tissue destruction and destruction of the liver cells. Approximately 8 weeks after infection *F. hepatica* reach the bile ducts, where they continue to grow and develop into adults, reaching full size by 12–14 weeks after infection (Anderson and Fairweather, 1995; Fairweather *et al*. 1999). Due to the massive damage of the migrating juvenile stages of the parasite, an ideal fasciolicidal drug should be active against both parasite stages, as is triclabendazole (Keiser *et al*. 2005; Fairweather, 2009).

F. hepatica in vitro *assays*

Newly encysted juveniles, young and adult worms can be cultured *in vitro* (Fig. 5, Table 1). An assay based on newly excysted juveniles offers the advantage that, as for the schistosomula assay described above, a mammalian host is not required. Metacarcariae excyst when exposed to appropriate stimuli (Dixon, 1966). Commonly used SOPs include exposure of metacercariae to an activation medium followed by incubation in an emergence medium. For example, the following protocol has been described by McGonigle and colleagues: metacercariae are excysted by incubation in 0·5% sodium hypochlorite for 20 min prior to centrifugation (2000 *g* for 2 min), washing in distilled water and incubation in 0·5% sodium bicarbonate, 0·4% sodium chloride, 0·2% sodium tauroglycholate, 0·07% concentrated HCl and 0·006% l-cysteine for up to 3 h at 37 °C (McGonigle *et al*. 2008). Excysted juveniles are maintained in nutritive culture medium, such as

Table 2. *In vivo* drug screening: rodent host, infection and evaluation procedures of juvenile and adult *S. mansoni*, *F. hepatica* and *E. caproni* assays

Trematode	Animal host	Route of infection and infective dose	Chemotherapeutic studies on juvenile worms	Chemotherapeutic studies on adult worms	Evaluation of assay
Schistosoma mansoni	**Mouse**, hamster, rabbit	Skin penetration or subcutaneous or intraperitonal injection of ~80 cercariae	Treatment 21 days after infection and collection of worms from the livers and mesenteric veins several days/weeks after treatment	Treatment 49 days after infection and collection of worms from the livers and mesenteric veins several days/weeks after treatment	Worm burden reduction, egg count reduction, morphology, motility of worms, SEM and TEM studies
Fasciola hepatica	**Rat**, rabbit, hamster (sheep serve as larger, non-rodent hosts)	Oral infection with 20–30 metacercariae (rat) or ~250 metacercariae (sheep)	Treatment 3–5 weeks after infection and collection of worms from livers several days/weeks after treatment	Treatment 8–12 weeks post-infection and collection of worms from bile ducts several days/weeks after treatment	
Echinostoma caproni	**Mouse**, hamster	Oral infection with 25–50 metacercariae	NA	Treatment 10–14 days post-infection and collection of worms from the intestines several days/weeks after treatment	

NA Not applicable

RPMI 1640, supplemented with penicillin and streptomycin and possibly serum or red blood cells (Ibarra and Jenkins, 1984). *In vitro* assays based on newly excysted flukes have been used to study the fasciolicidal activities of investigational compounds; for example, 20 plant extracts were recently evaluated using this parasite stage (Vera-Montenegro *et al*. 2008). Nonetheless, newly excysted flukes might have different drug sensitivities when compared to juvenile and adult flukes: a study which compared the fasciolicidal activity of known fasciolicides demonstrated that newly excysted flukes were unaffected by most of these compounds (Ibarra and Jenkins, 1984).

Assays based on juvenile or adult *Fasciola* are more often used to study fasciolicidal properties of investigational drugs *in vitro*. The main disadvantages of these assays are that only few flukes can be recovered from the laboratory rat, which is the main rodent host. Rats tolerate an infective dose of 20–30 metacercariae, yielding between 2 and 13 mature flukes (Chapman and Mitchell, 1982). In addition, as mentioned above, *Fasciola* spp. grow for 8–12 weeks until they are fully mature. Hence, these assays are expensive and time consuming. Another possibility to obtain parasite material in a more efficient and less costly manner in endemic countries is to collect worms from animals slaughtered in abattoirs (Shalaby *et al*. 2009). Juvenile or adult *Fasciola* are incubated in the presence of RPMI 1640 or NTCT 135, supplemented with antibiotics and the investigational drug (Anderson and Fairweather, 1995; Keiser *et al*. 2006). Though supplementation of the medium with serum is recommended for long term culture of *Fasciola* spp. (longer than 4 days), serum substitution may not be advised for *Fasciola* drug activity studies, as many drugs bind strongly to serum proteins (Jenkins *et al*. 1987). Fasciolicidal drugs such as rafoxanide or bithionol showed a decreased activity *in vitro* in the presence of serum compared to serumless medium (Jenkins *et al*. 1987). The presence of bile in the incubation medium has also been shown to influence the uptake pattern of fasciolicidal drugs, such as triclabendazole or albendazole (Alvarez *et al*. 2004). As described above for the schistosomes, fasciolicidal properties are examined documenting motility, morphological changes and death. To my knowledge metabolic markers have not been used in *Fasciola* drug sensitivity assays. Drug effects have also been well documented using SEM and transmission electron microscopy (TEM) (Anderson and Fairweather, 1995; McConville *et al*. 2006; Keiser and Morson, 2008). For example, Fig. 6a shows a SEM image of the anterior region of a *F. hepatica*. We have recently described that the semi-synthetic artemisinin derivatives artemether and artesunate, which are essential components of malaria therapy (White, 2008) and synthetic derivatives possess a broad range of trematodicidal activities (Keiser and

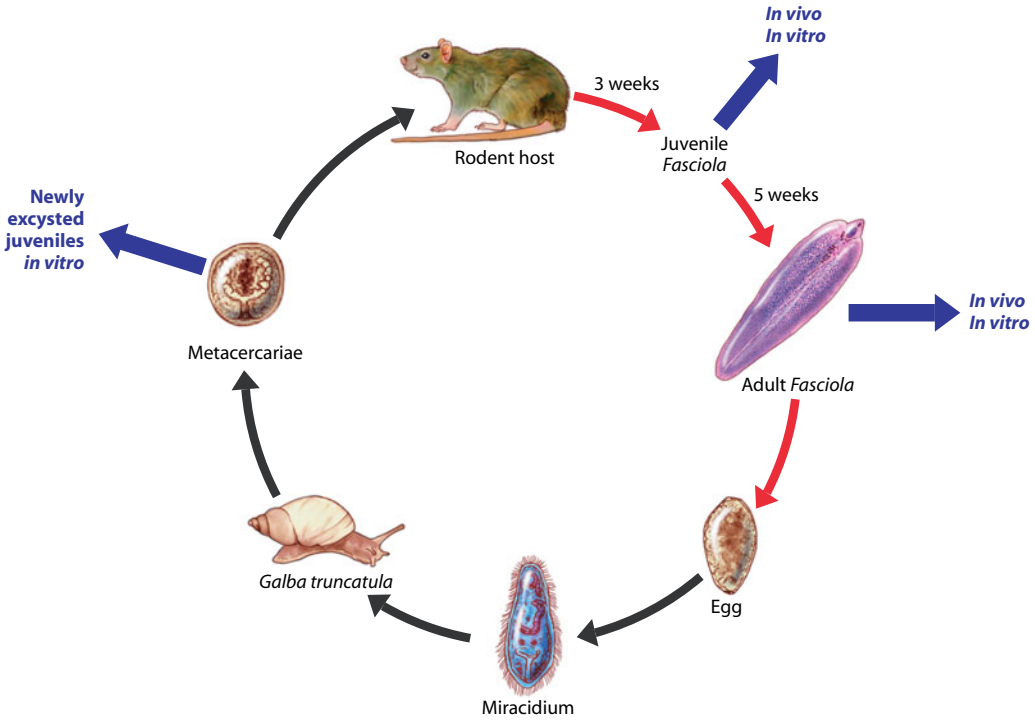

Fig. 5. Life cycle of *F. hepatica* highlighting collection points for *in vitro* and *in vivo* chemotherapeutic studies. Maturation of parasite occurs within final host (red arrows).

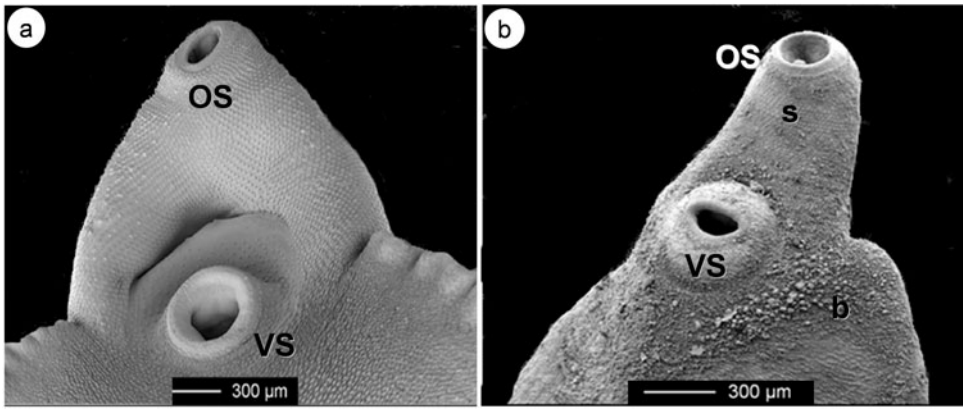

Fig. 6. *Fasciola hepatica* – SEM observations. (a) anterior region of an adult *F. hepatica* recovered from a rat (OS: oral sucker, VS: ventral sucker). (b) Blebbing (b) and sloughing (s) visible near the ventral sucker (VS) on a *F. hepatica* recovered from a rat 48 h post-treatment with a single oral dose of 200 mg/kg artemether.

Utzinger, 2007; Utzinger *et al*. 2007). Fig. 6b depicts the head region of a *Fasciola* fluke 48 h post-treatment with a single 200 mg/kg oral dose of artemether, revealing blebbing, sloughing and roughening of the tegumental surface (Keiser and Morson, 2008).

F. hepatica in vivo *assays*

F. hepatica can be grown in different laboratory animals but, as mentioned above, the rat is the most commonly used rodent to study *F. hepatica in vivo* (Fig. 5, Table 2). No significant difference was observed between different rats strains infected with *F. hepatica* with regard to worm burden (Chapman and Mitchell, 1982). Rats, commonly infected with 20–30 metacercariae, harbour between 2–13 adult flukes (Chapman and Mitchell, 1982). The laboratory mouse is not a suitable model to grow adult *F. hepatica*, as mortality of mice is high and the flukes do not fully develop in this model (Chapman and Mitchell,1982; Smyth and Halton, 1983). The infectivity of *F. gigantica* was found to be low in Wistar rats (Itagaki *et al*. 1994). Rabbits, the rat-like hamster, *Tscherskia triton*, and sheep have also been used for studies of experimental fascioliasis (Terasaki *et al*. 2003; McConville *et al*. 2009).

In chemotherapeutic studies, groups of rats or sheep, which had been experimentally infected with *F. hepatica* for 3–5 weeks (juvenile infection) and 8–12 weeks (adult infection) or sheep harbouring a

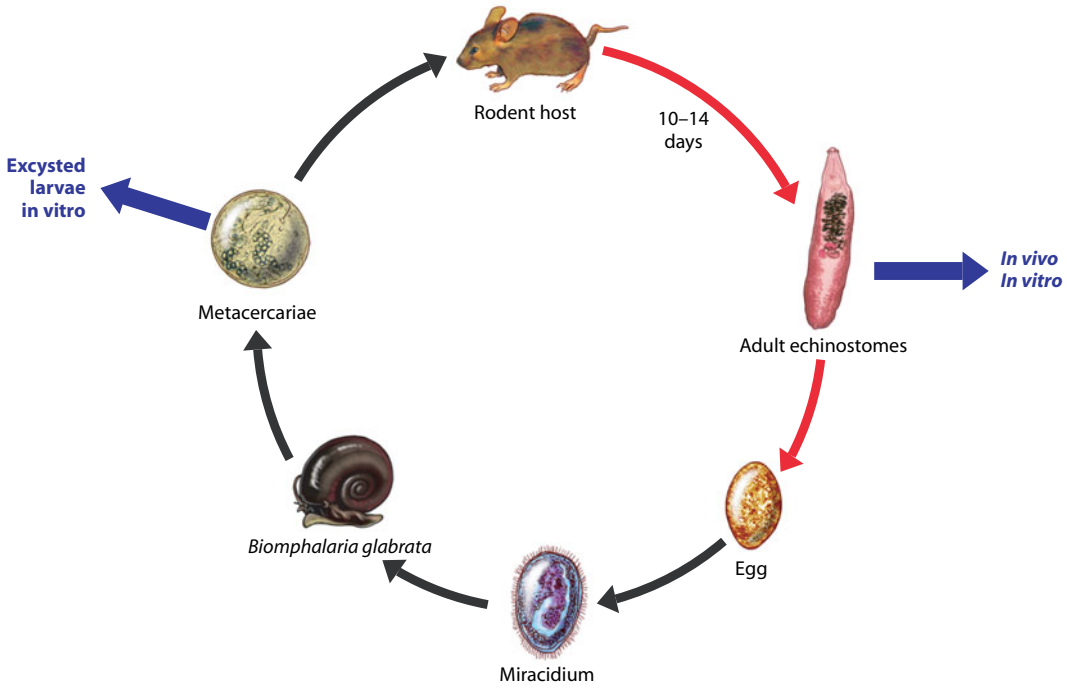

Fig. 7. Life cycle of *E. caproni* highlighting collection points for *in vitro* and *in vivo* chemotherapeutic studies. Maturation of parasite occurs within final host (red arrow).

natural infection are treated with the investigational drugs and the numbers of flukes present in the livers and bile ducts are counted several weeks post treatment. Groups of untreated animals serve as controls (Keiser *et al.* 2006, 2008; McConville *et al.* 2009). Besides the effect of the drug on the *Fasciola* worm burden, the egg count reduction and the effect of the drug on the morphology, tegument or intestinal structures are studied (McKinstry *et al.* 2003; Keiser and Morson, 2008; McConville *et al.* 2009).

Revisiting the artemisinins, the fasciolicidal properties of artemether were thoroughly studied in *F. hepatica*-infected rats (both a triclabendazole-resistant and -sensitive isolate) and sheep harbouring a natural *F. hepatica* infection: single oral artemether at 200 mg/kg achieved a complete cure in rats (Keiser *et al.* 2006, 2007), while a worm burden reduction of 91% was recorded in sheep following a single intramuscular administration of 160 mg/kg artemether (Keiser *et al.* 2008).

ECHINOSTOMA CAPRONI

The third example is the intestinal fluke *E. caproni*, which has been widely used as an experimental model in different areas of research, as its life cycle is easy to maintain in the laboratory (Fried and Huffman, 1996). Laboratories, which maintain this life cycle are depicted in Fig. 1. Echinostomes invade domestic animals, wildlife or humans (Toledo and Fried, 2005). The rodent and the avian fluke *E. caproni* cycles between *B. glabrata* snails and laboratory mice (Fig. 7) (Fried and Huffman, 1996). Briefly, mice are infected orally with 25–50 metacercariae, though up to 300 metacercariae are tolerated (Platt, 2009). An adult infection is established ~2 weeks after infection. For life cycle maintenance, eggs can be removed either directly from the worms collected from mice intestines or faeces. After embryonation of eggs for about 14–17 days at room temperature miracidia develop. *Biomphalaria glabrata* are exposed to fully developed eggs or miracidia (Idris and Fried, 1996). Four to six weeks after infection of snails, most snails in the aquarium contain hundreds of metacercariae in the pericardial kidney region (Fried and Huffman, 1996).

E. caproni in vitro *assay*

Encysted metacercariae of *E. caproni* can be excysted in a trypsin-bile salts-cysteine medium (Table 1) (Saxton *et al.* 2008; Fried and Peoples, 2009). Excysted larvae have been mainly used in *in vitro* culture studies and might also prove useful for chemotherapeutic, biological or physiological studies as reviewed elsewhere (Fried, 1994). Excysted larvae are cultured in a defined medium such as NCTC 135 supplemented with antibiotics and different types of natural products such as serum, egg yolk or mucus (Fried, 2000).

Several studies have examined the echinostomicidal properties of investigational drugs using adult echinostomes *in vitro* (Leger *et al.* 1973; Leger and Notteghem, 1975; Keiser *et al.* 2006). Though adult echinostomes maintained *in vitro* deteriorate quickly, possibly due to microbial contamination and/or

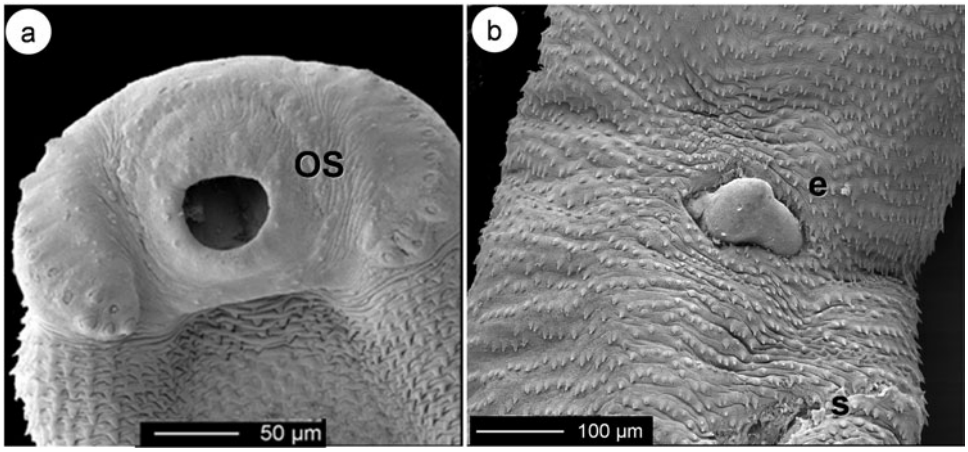

Fig 8. *Echinostoma caproni* – SEM observations. (a) anterior region of an adult *E. caproni* recovered from a mouse. (b) eruption (e) and sloughing (s) visible on the ventral surface of an *E. caproni* 4 h post-treatment with a single oral dose of 700 mg/kg artesunate.

worm autolysis (Fried, 2000), echinostomes can be maintained in nutritive media such as RPMI 1640 ideally supplemented with additional glucose (e.g. 1%) for several days (Leger *et al*. 1973). As described above worms incubated in the presence of drugs are analyzed for occurrence of death, motility disturbances or morphological changes.

E. caproni in vivo *assay*

While there is a high degree of compatibility between *E. caproni* and mice and hamsters, rats show a low degree of compatibility with *E. caproni* (Toledo and Fried, 2005). *E. caproni* can survive for more than 20 weeks in mice and hamsters (Toledo and Fried, 2005). The laboratory mouse is most commonly used to study *E. caproni in vivo* (Table 2). As adult infections are established already by 10–14 d after infection, this model allows for quicker patent infections in the definitive hosts than the *F. hepatica* or *S. mansoni* assays described above. Hence, results are obtained rapidly and cost-effectively in the *E. caproni*-mouse model. *In vivo* studies in *Echinostoma* spp. infected mice, with various test compounds such as the benzimidazoles, praziquantel, the artemisinins or tribendimidine against echinostomes in mice have been summarized recently (Saric *et al*. 2009). For example, *E. caproni*-infected mice were cured following a single oral dose of 700 mg/kg artesunate (Keiser *et al*. 2006). In Fig. 8a the head region of an untreated *E. caproni* is depicted. Four h after treatment with a single 700 mg/kg oral dose of artesunate eruption and sloughing was observed on the ventral surface of an *E. caproni* fluke (Fig. 8b) (Keiser *et al*. 2006).

CONCLUSION

Schistosomiasis and food-borne trematodiases are chronic parasitic diseases affecting millions of people, mostly in the developing world. The number of drugs available to treat these diseases is surprisingly small and preliminary reports of resistance in the field and laboratory highlight the need to develop new trematodicidal drugs (Colley *et al*. 2001; Caffrey 2007; Keiser and Utzinger, 2009). *In vitro* and *in vivo* whole parasite screens represent essential components of the trematodicidal drug discovery cascade in particular as molecular-based target drug screening approaches are still very limited. Nonetheless, publications of latest draft genome sequences e.g. for schistosomes (Berriman *et al*. 2009; Liu *et al*. 2009) will provide new insights into the biology of trematodes and offer an opportunity for identification of potential drug targets. For example, 71,028 compounds were recently screened for thioredoxin/glutathione reductase and peroxiredoxin activity, principal components of the defence system of schistosomes (Cioli *et al*. 2008; Simeonov *et al*. 2008). Efforts to enter novel molecular targets in the trematodicidal drug-discovery process should go hand-in-hand with an improvement of existing whole organism screens. Validated, robust, high throughput *in vitro* systems are needed to boost the discovery pipeline for these diseases (Colley *et al*. 2001). Difficulties still need to be overcome in the basic cultivation of trematodes. In addition, phenotypic assessment of treated parasites is still the method of choice in *in vitro* drug screens and the development of simple readouts that are amenable to automated analysis (e.g. calorimetric analysis, viability markers) would be a great step forward.

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