Echinococcus metacestodes as laboratory models for the screening of drugs against cestodes and trematodes

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SUMMARY

Among the cestodes, *Echinococcus granulosus*, *Echinococcus multilocularis* and *Taenia solium* represent the most dangerous parasites. Their larval stages cause the diseases cystic echinococcosis (CE), alveolar echincoccosis (AE) and cysticercosis, respectively, which exhibit considerable medical and veterinary health concerns with a profound economic impact. Others caused by other cestodes, such as species of the genera *Mesocestoides* and *Hymenolepis*, are relatively rare in humans. In this review, we will focus on *E. granulosus* and *E. multilocularis* metacestode laboratory models and will review the use of these models in the search for novel drugs that could be employed for chemotherapeutic treatment of echinococcosis. Clearly, improved therapeutic drugs are needed for the treatment of AE and CE, and this can only be achieved through the development of medium-to-high throughput screening approaches. The most recent achievements in the *in vitro* culture and genetic manipulation of *E. multilocularis* cells and metacestodes, and the accessability of the *E. multilocularis* genome and EST sequence information, have rendered the *E. multilocularis* model uniquely suited for studies on drug-efficacy and drug target identification. This could lead to the development of novel compounds for the use in chemotherapy against echinococcosis, and possibly against diseases caused by other cestodes, and potentially also trematodes.

Key words: Cystic echinococcosis (CE), alveolar echinococcosis (AE), *Echinoccoccus granulosus*, *Echinoccoccus multilocularis*, *in vitro* culture, chemotherapy.

INTRODUCTION

The genus *Echinococcus* includes seven to nine described species (Nakao *et al*. 2007; Varcasia *et al*. 2008). Of these, *Echinococcus multilocularis* (the small fox tapewom) is the most pathogenic, and *E. granulosus* (the small dog tapeworm) represents the most common species (Thompson, 1986). Both cause life-threatening diseases of serious public health and economic concern worldwide (McManus *et al*. 2003). Alveolar echinococcosis (AE), caused by *E. multilocularis*, is largely restricted to the Northern hemisphere, such as Central Asia, Russia, Western China, Europe and Japan. Infections with *E. granulosus*, the causative agent of cystic echinococcosis (CE), occur globally, and mostly in the Mediterranean area, Central Europe, South America, Africa, Central Asia, and CE exists as an imported disease in Western Europe and the USA (Schantz *et al*. 1995; Eckert and Deplazes, 2004). 3·6 million disability-adjusted life years (DALYs) are lost due to CE (Craig *et al*. 2007), showing that the impact of the disease is comparable to onchocercosis and African trypanosomosis (Budke *et al*. 2006). AE and CE are diseases of communities that often lack essential resources, thus the development of new drugs against these diseases has not been a major focus of the pharmaceutical industry. First, because the population affected and the number of cases as such do not represent a promising market. Secondly, there has been an inherent lack of *in vitro* culture systems that would allow cost-effective high-throughput drug screening. However, the recent achievements in the establishment of *in vitro* cultivation systems, especially for *E. multilocularis* metacestodes, has now opened the door for medium-to-high-throughput drug screening possibilities. These developments could have considerable impact not only for the treatment of echinococcosis, but also for other diseases caused by cestodes such as *Taenia, Hymenolepis, Mesocestoides* and *Spirometra* and, potentially, trematodes.

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ECHINOCOCCUS: BIOLOGY AND DISEASE

E. multilocularis and *E. granulosus* share some distinct features in their life cycle. The adult worms live

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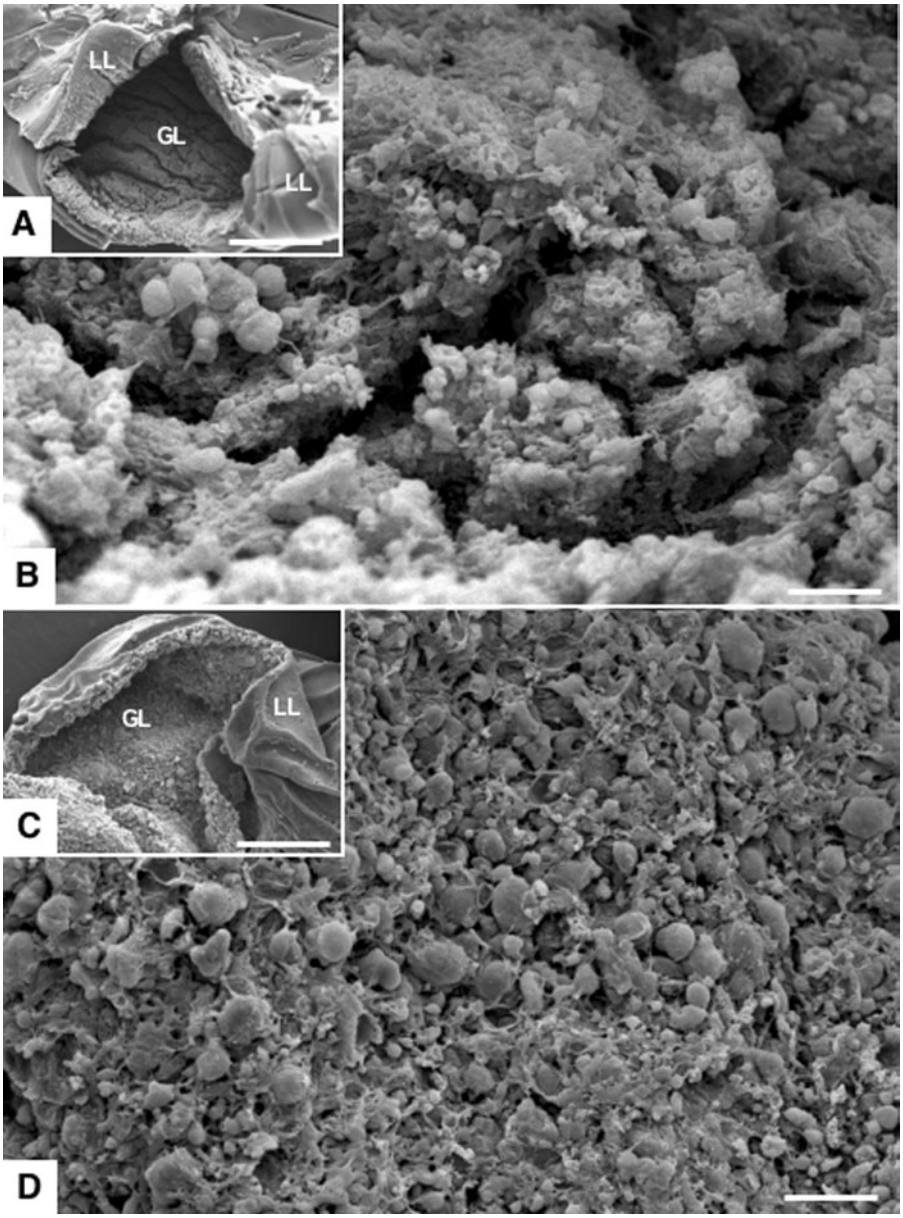

Fig. 1. SEM of *E. granulosus* (A, B) and *E. multilocularis* (C, D) metacestodes. A and C represent lower magnification views of broken vesicles, exposing both the acellular laminated layer (LL) and the germinal layer (GL); bars = 1·5 mm. B and D represent higher magnification views of the germinal layer of *E. granulosus* and *E. multilocularis*, respectively; bars = 400 μm.

in the intestine of their respective final host (dogs for *E. granulosus*, foxes, dogs and cats for *E. multilocularis*) where sexual reproduction and subsequent egg production takes place. Faecal shedding spreads the eggs into the environment, where they are accidentally taken up by suitable intermediate hosts, such as small rodents for *E. multilocularis*, and cattle and sheep for *E. granulosus*. These eggs contain the first larval stage, the oncosphere, and during stomach passage, the oncosphere is activated and leaves the protective egg. It actively penetrates the intestinal lining, and migrates via blood and lymphatic vessels to the visceral organs. These are primarily the liver for *E. multilocularis*, and the liver, lung and other target organs in the case of *E. granulosus*. There, these oncospheres develop into metacestodes, which represent the second larval stage. Within these metacestodes, protoscolex development takes place, and upon oral uptake by the respective final host, protoscoleces attach to the intestinal epithelium and develop into adult worms, thus concluding the life cycle (Rausch, 1995).

Metacestodes of both species are fluid-filled vesicles, and represent the disease-causing stage. They are structured into an inner cellular and an outer acellular compartment (Fig. 1). The outer, acellular surface of the metacestode is formed by the laminated layer, a carbohydrate-rich structure

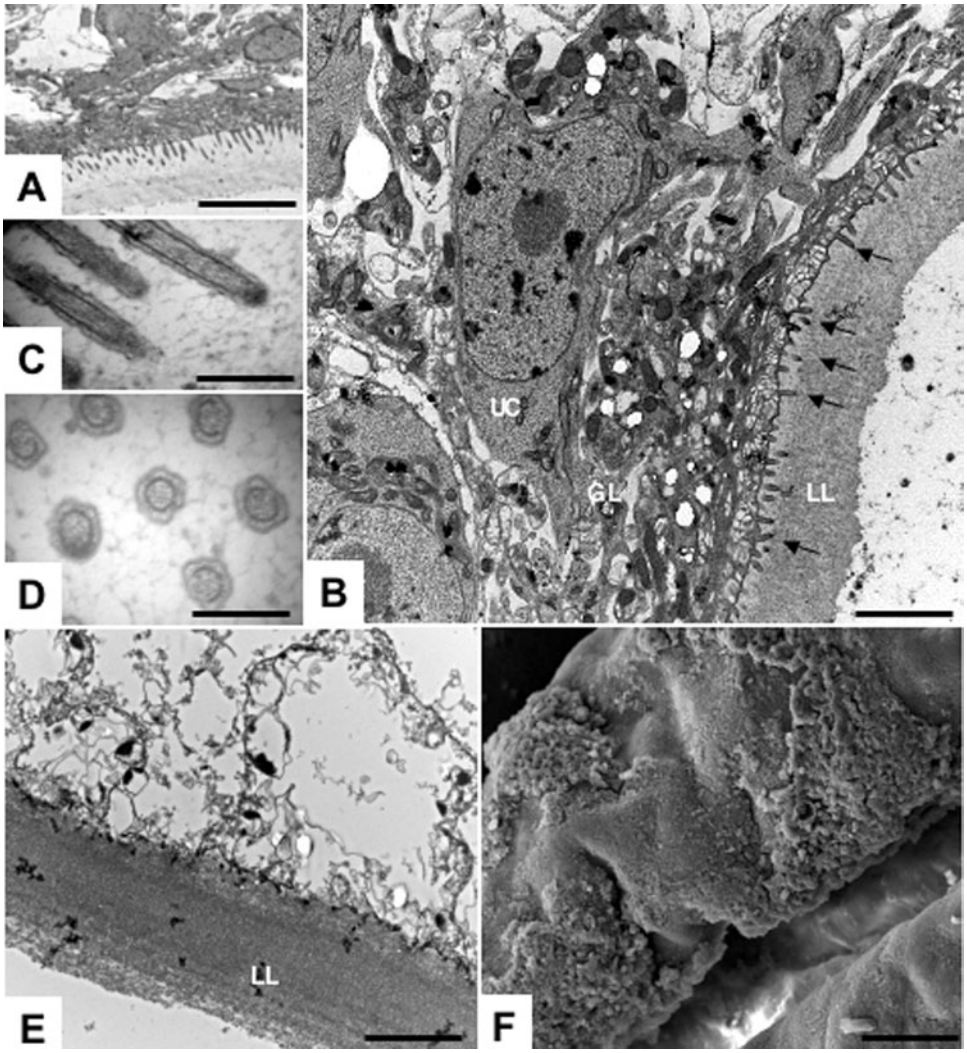

Fig. 2. TEM of *E. multilocularis* metacestodes cultured *in vitro*. A shows a low magnification view of a section through the metacestode wall, with laminated (LL) and germinal layer (GL); bar = 7 μm. B shows a higher magnification view, demonstrating LL, GL-tissue with different cell types including undifferentiated cells with a large nucleus and nucleolus (uc), and the tegument with microtriches (arrows); bar = 3·2 μm. C and D show higher magnification views of longitudinally sectioned (C; bar = 0·56 μm) and cross-sectioned (D; bar = 0·43 μm) microtriches revealing actin microfilaments as main structural components. Also note the filamentous meshwork of electron-dense thin filaments that is embedded into the matrix of the LL.

synthesized by the parasite. The laminated layer covers the entire metacestode surface, and is much more prominent in *E. granulosus* metacestodes (Morseth, 1967; Gottstein and Hemphill, 1997). The actual larval tissue is formed by the germinal layer, which itself is composed of a population of different cell types (Figs. 1 and 2). One part consists of the tegument, which is directly associated with the inner surface of the laminated layer. It is characterized by microvilli-like extensions, microtriches, which protrude into the matrix of the laminated layer and thereby increase the resorbing surface of the parasite (Fig. 2). In addition, the germinal layer contains highly differentiated cell types including connective tissue, muscle cells, and glycogen storage cells, as well as many undifferentiated cells (Fig. 2).

The fully mature *E. granulosus* metacestode (i.e. hydatid cyst) is a single-chambered or septate unilocular cyst that shows expansive growth and thereby causes compression of neighbouring tissue, tissue damage and organ dysfunction (Kern, 2006). Accidental or traumatic cyst rupture can result in release of cyst fluid and dissemination of protoscoleces, leading to anaphylactic reactions and metastases (secondary echinococcosis) (Stey and Jost, 1993). *E. granulosus* metacestodes are surrounded by a host fibrous capsule, the adventitial layer, which is composed of host connective tissue.

In *E. multilocularis* infection, metacestode development exhibits different characteristics. There is no limiting host-tissue barrier comparable to the adventitial layer. The metacestode tissue represents a multivesicular structure that is reproducing

asexually, by formation and budding of daughter vesicles, with progressive tumour-like growth (Ali-Khan *et al*. 1983). This leads to the formation of a large and heterogenous parasitic mass that is intermingled with host connective tissue, proliferative in the periphery, and often necrotic in the centre (Gottstein and Hemphill, 1997). Metastases formation may occur in other organs due to the release of germinal layer cells into the blood or lymph system (Ali-Khan *et al*. 1983; Eckert *et al*. 1983; Mehlhorn *et al*. 1983). Thus, AE can resemble a benign malignant tumour (Vuitton, 2009). Protoscolex development in humans has been rarely observed.

BENZIMIDAZOLES FOR THE CHEMOTHERAPEUTIC TREATMENT OF CYSTIC ECHINOCOCCOSIS (CE) AND ALVEOLAR ECHINOCOCCOSIS (AE)

For many years, the preferred treatment strategy for CE has been radical resection of the parasite mass (Kern, 2003, 2006). Other options include image-guide percutaneous treatment (PAIR = puncture, aspiration, injection, reaspiration) (Brunetti *et al*. 2004) and chemotherapy (El-On, 2002). Both surgery and PAIR are always accompanied by chemotherapy and, for inoperable cases, chemotherapy remains the only option. These cases include patients exhibiting cysts in crucial organs such as spine, pelvis and other sites that are not easily accessible, or patients with multiple cysts in several organs. The currently used drugs are mebendazole and albendazole. These drugs clearly have the potential to kill metacestodes, and to cure the patients. Benzimidazoles exert their action by binding to tubulin, inducing microtubule depolymerization and inhibiting polymerization of this essential cytoskeletal element (reviewed by Hemphill and Müller, 2009). However, the efficacy of these benzimidazoles depends on size and type of cyst (benzimidazoles are more effective against smaller cysts), on the age of the patient (these drugs work better in younger than in older patients), and on which organ is affected (e.g. cysts localized in bones are less susceptible to chemotherapy than those in liver and lungs). The duration of treatment plays a crucial role, with prolonged drug administration producing the most favourable results for the patients (Horton, 1997; Franchi *et al*. 1999; Vutova *et al*. 1999). Praziquantel, a heterocyclic pyrazinoisoquinoline derivative, has been proposed to be used alongside benzimidazoles in CE-patients. Praziquantel is well tolerated, less toxic and better absorbed than albendazole. Praziquantel is used against the adult stages of *Echinococcus* and many other cestodes, and was shown to exhibit a high efficacy against protoscoleces (Morris *et al*. 1986) and metacestodes in animal experiments (Urrea-Paris *et al*. 1999, 2001). The mode of action of praziquantel is a matter of debate. Respective studies were mostly carried out with schistosomes, where praziquantel represents the only drug that is currently marketed. Possibilities of praziquantel toxicity include its actions on nucleoside uptake (Angelucci *et al*. 2007), inhibition of phospho-inositide turnover (Wiest *et al*. 1992), binding to parasite actin (Tallima and El Ridi, 2007) and the parasite myosin light chain, and possibly inhibiting its functional activity (Gnanasekar *et al*. 2009), interference in glutathione-S-transferase activity (McTigue *et al*. 1995), and stimulation of Ca^{2+} entry through voltage-operated Ca^{2+} channels (VOCCs) (Kohn *et al*. 2003; Jeziorski and Greenberg, 2006). The most compelling evidence for the involvement of VOCCs was recently provided by Nogi *et al*. (2009): they used the free-living flatworm *Dugesia japonicum* and investigated regeneration of fragments excised from this planarian that have the ability to reform a complete body plan, an ability that is driven by a totipotent population of stem cells called neoblasts. In the presence of praziquantel, however, this regeneration process yielded complete duplication of the entire antierior-posterior axis, resulting in two-headed organisms with duplicated nervous and organ systems. This effect of praziquantel was selectively ablated by *in vivo* RNAi of VOCC beta-subunits, but not by knock-down of alpha-subunits. At higher doses of praziquantel, knock-down of VOCC beta-subunit also conferred resistance to praziquantel, confirming the critical involvement of this beta subunit in the action of the drug.

A combined treatment regimen with albendazole and praziquantel given during the month prior to surgery of *E. granulosus*-infected patients increased the number of human patients with non-viable protoscoleces as compared to therapy with albendazole alone (Cobo *et al*. 1998). Thus, praziquantel is regarded as useful in cases where cyst content is spilled during surgery.

For the treatment of AE, surgery and chemotherapy are the two only treatment options (Ammann and Eckert, 1995). Spontaneous cure of AE, leading to calcified lesions, is possible, but it is not known how commonly this occurs (Gottstein and Hemphill, 1997; Vuitton, 2009). Radical surgery of viable lesions is carried out if possible, but can be difficult to achieve, and only about one third of all cases are actually operated on. In contrast to *E. granulosus* metacestodes, *E. multilocularis* metacestodes are almost exclusively located in the liver. However, metastases can occur, also involving distant sites including lungs, spleen and brain. Chemotherapy should last for at least 2 years post-surgery, and monitoring of patients should be continued for 10 years. Inoperable cases must undergo long-term chemotherapy, often life-long (Reuter *et al*. 2000; 2004; Vuitton, 2009). The experiences with long-term use of benzimidazoles has had an enormous

impact on the use of surgery. Generally, palliative surgery should be avoided and replaced by benzimidazole treatment alone or combined with percutaneous or perendoscopic interventions to treat biliary or vascular complications (Bresson-Hadni *et al*. 2000, 2006; Vuitton, 2009). Extensive animal experimentation and clinical experience in human patients have both demonstrated that albendazole and mebendazole exhibit a parasitostatic rather than parasiticidal effect against *E. multilocularis* metacestodes (Reuter *et al*. 2004; Vuitton, 2009). Thus benzimidazoles only prevent parasite growth, and the recurrence rates after interruption of therapy are high. Nevertheless, clinical studies have shown that chemotherapy has significantly increased the 10-year survival rate of inoperable or non-radically operated AE patients from 6–25% to 80–83% (Hemphill *et al*. 2007).

Benzimidazoles are generally well tolerated, but problems can occur. Adverse reactions include hepatotoxicity, alopoecia, gastrointestinal disturbances and leukopaenia, which is sometimes severe and irreversible (Horton, 1997). Risks also include embryotoxicity and teratogenicity. In order to improve bioavailability, liposome-entrapped formulations and emulsions of albendazole have been tested (Wen *et al*. 1996; Chai *et al*. 2004). The use of cimetidine is discouraged, since it increases intestinal absorption (Wen *et al*. 1996; Schipper *et al*. 2000), which in turn could result in toxic effects.

Major problems associated with benzimdazoles are the intra- and inter-individual variations of the pharmacokinetics, which make it necessary to measure albendazole sulphoxide and mebendazole plasma levels on a regular basis in order to adjust drug dosage and to avoid toxicity (Vuitton, 2009). This is only possible in specialized facilities, and thus precludes the use of benzimidazoles in those endemic areas where efficient drugs would be mostly needed. In addition, it is unknown whether there is a stage-specific response to exposure to benzimidazoles, and the actual concentration at the site where the drug exerts its action is not known. These pharmacological uncertainties, together with the difficulties in assessing metacestode viability in humans, show that novel and improved chemotherapeutical tools are needed in order to optimize the treatment of CE and AE.

THE ROLE OF *IN VITRO* CULTURE IN DRUG DISCOVERY

In order to identify novel potential alternatives for chemotherapy against echinococcosis, the strategy used most commonly has been whole organism screening of *E. multilocularis* and *E. granulosus* metacestodes and/or protoscoleces. In most cases, researchers have focused on broad-spectrum anti-infective agents and anti-cancer compounds, many of which also exhibited reasonable efficacy against *Echinococcus* (Hemphill and Müller, 2009).

In vitro chemotherapy studies on CE have mostly, but not exclusively, been carried out on protoscoleces, since these are easily obtained from cysts of an infected animal. The metabolism, and thus drug-susceptibility, of *E. granulosus* protoscoleces might however be different from the metacestode stage tissue. As an example, praziquantel is highly active against adult tapeworms and against protoscoleces, but only of limited efficacy against metacestodes. Conversely, albendazole is less active against protoscoleces, but of significantly higher efficacy against metacestodes (Taylor *et al*. 1989).

Infection of laboratory animals with *E. granulosus* protoscoleces results in the development of metacestodes, which mimics the process of secondary hydatid disease in humans, and this differentiation process can also be achieved *in vitro* (Walker *et al*. 2004). *E. granulosus* metacestodes do not proliferate *in vitro* but, as *in vivo*, show a marked increase in size. Thus, extensive animal experimentation is necessary for constant supply of metacestode material. In contrast, *E. multilocularis* metacestodes, besides increasing in size, also proliferate asexually and, provided with the corresponding nutrients and growth factors, will form new vesicles either endogenously (from within the germinal layer) or exogenously (by budding of daughter metacestodes from older vesicles; Fig. 3). Thus, the fact that *E. multilocularis* metacestodes proliferate *in vitro* has rendered this model suitable for the experimental assessment of the effects of chemotherapeutically interesting compounds.

Historically, the *in vitro* culture of *E. multilocularis* metacestodes was achieved by dissection of infected rodents and placing small pieces of infected tissue or vesicle suspension into a suitable culture medium at 37 °C. After a few weeks, newly formed metacestodes emerged, which were infective when re-introduced back into rodents (Hemphill and Gottstein, 1995). These were co-culture systems, as parasites could be maintained for several months in the presence of feeder cells, and in the absence of feeder cells, metacestodes degenerated within a few weeks. Jura *et al*. (1996) introduced a second *in vitro* co-culture system, which was based on co-incubation of homogenized parasite tissue obtained from infected rats with primary rat hepatocytes embedded in a collagen layer. Although useful in many instances (reviewed in Hemphill *et al*. 2002, 2007; Brehm and Spilitois, 2008*a*), these co-culture systems provided only moderate yields and only a very limited number of drugs could be studied. Thus, the amount of metacestodes that could be generated was not sufficient for larger scale investigations.

Recently, Spiliotis and Brehm (2008) have overcome this problem and established a large-scale cultivation system which utilizes homogenized

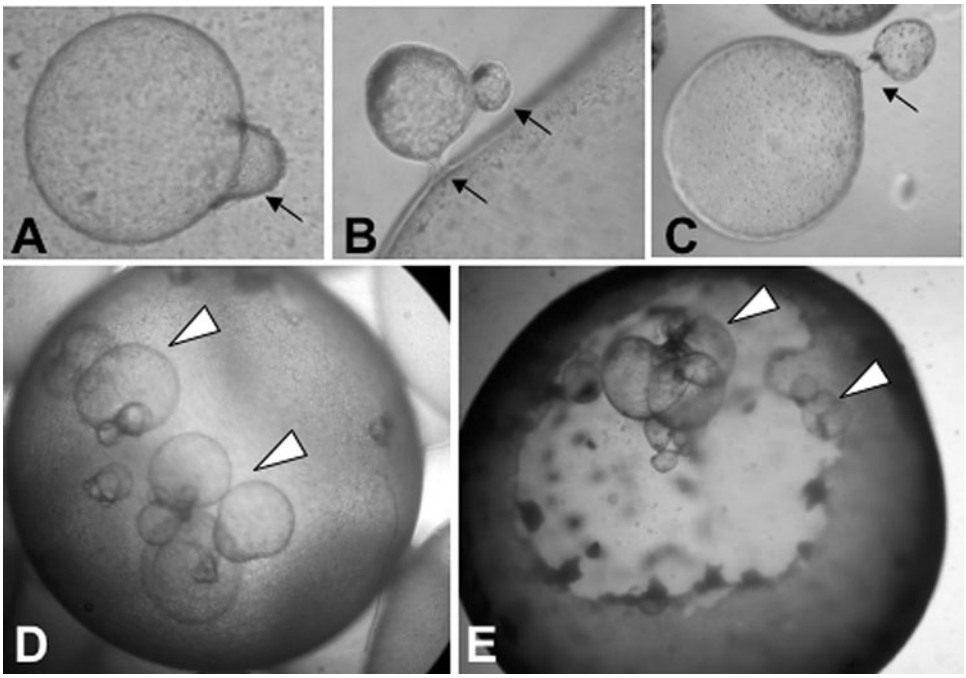

Fig. 3. Exogenous and endogenous proliferation of *E. multilocularis* metacestodes *in vitro*. Exogenous outgrowth of a newly formed vesicle (arrows in A–B) from a pre-exisiting parent vesicle appears to be followed by budding off of the daughter vesicle (C). Vesicles are also formed within older metacestodes, most likely by emerging out of the germinal layer, and these smaller metacestodes are released upon desintegration of the older metacestode (D, E).

metacestode tissue that is incubated in liquid culture together with rat hepatoma feeder cells. Using this system, high numbers of metacestodes with comparable sizes can be generated within a few weeks of incubation. Thus, a major task of feeder cells appears to be the provision and secretion of growth factors that are needed by the parasites to undergo proliferation, growth and differentiation. *E. multilocularis* metacestodes possess several receptors that interact with human epidermal growth factor (EGF), insulin, transforming growth factor-beta and steroid hormones (Brehm *et al.* 2006; Brehm and Spiliotis, 2008*b*), and these most likely allow the parasite to communicate with its host and adapt to alterations in living conditions, regulating gene expression to its favour. Of course, these receptors, and associated signalling pathways within the parasite, represent interesting targets for potential chemotherapeutical intervention (Brehm *et al.* 2006; Gelmedin *et al.* 2008). In addition, Spiliotis *et al.* (2004) developed the methodology to culture *E. multilocularis* metacestodes axenically, in the absence of feeder cells, under reducing and anaerobic conditions. In the presence of oxygen, these metacestodes degenerate within few weeks (Spilitois *et al.* 2004), illustrating the high sensitivity of *E. multilocularis* metacestodes to oxygen intermediates. The specific adaptation of the mitochondrial respiratory system to anaerobic environments has been shown also for *E. multilocularis* protoscoleces (Matsumoto *et al.* 2008). Thus, another important task of feeder cells is to remove toxic oxygen intermediates (Brehm and Spiliotis, 2008*a*).

METHODS FOR THE ASSESSMENT OF ANTI-ECHINOCOCCAL DRUG CANDIDATES

By using animal experimentation, the primary assessment of anti-echinococcal drug candidates has often been performed in mice or gerbils, through evaluation of parasite mass and/or health parameters of the host. This has led to the extensive use of animal experimentation, but has often yielded inconclusive results (reviewed in Siles-Lucas and Hemphill, 2002). More recently, *in vitro* cultured *Echinococcus* metacestodes have been increasingly used for the primary assessment of drug susceptibilities (Hemphill *et al.* 2002, 2007).

A major problem associated with drug-efficacy assessment has always been the monitoring of the actual metacestode viability following *in vitro* drug treatment. In a primary evaluation, the effects of *in vitro* drug treatment can be assessed mainly by visual inspection of morphological alterations and light-microscopy. SEM and TEM have also been used extensively to investigate tissue damage in more detail (Ingold *et al.* 1999; Stettler *et al.* 2003; Naguleswaran *et al.* 2006; Spicher *et al.* 2008*a*; see also Fig. 2E, F), and nuclear magnetic resonance spectroscopy (NMR) has been applied to investigate metabolic changes imposed upon the parasites by drug treatments (Ingold *et al.* 1999). Others have

attempted to assess parasite viability and growth by the quantification of the expression of molecular marker genes such as 14-3-3 and II/3-10 (Matsumoto *et al*. 2006). Visual inspection relies on subjective observations and thus requires experienced personnel, while other techniques such as SEM, TEM, NMR and RT-real time PCR are intrinsically time-consuming and expensive. Although these approaches have lead to the identification of several compounds with reasonable activities in the micromolar range *in vitro* (see below), it has not been possible to implement these techniques in a cost-effective manner at a larger scale, and they have not always produced conclusive results. For instance, *in vitro* culture of *E. multilocularis* metacestodes in the presence of albendazole sulphoxide for 14 days resulted in altered composition of vesicle fluid metabolites as assessed by NMR, and complete destruction of the metacestode structural integrity as visualized by SEM and TEM (Ingold *et al*. 1999). However, bioassay (re-inoculation of treated parasite material into mice) subsequently showed that the parasite had not been killed completely (Stettler *et al*. 2003). This was confirmed by Reuter *et al*. (2006), and illustrates the difficulties in viability assessment following drug treatment.

Although several potentially interesting compounds were identified, and combinations of some of these drugs with albendazole led to slightly improved treatment efficacy in experimentally infected mice or gerbils, none of these compounds exhibited improved activities compared to albendazole (reviewed in Hemphill *et al*. 2007). Thus, many more drugs and/or compound classes should be investigated, and there is an urgent need for a reliable, but also easy-to-handle and rapid *in vitro* drug screening assay for the identification of chemotherapeutically interesting compounds *in vitro*.

Attempts to develop screening assays based on the use of vital dyes employed for the assessment of cell viability, such as Alamar blue and Trypan blue, were undertaken. They worked well for protoscolex viability asessment (Walker *et al*. 2004), but they were not practical when used with metacestodes, as these dyes tend to bind to components of the laminated layer (M. Spicher *et al*. unpublished observations). The MTT viability assay, which is based on the activity of mitochondrial reductase that reduces yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide to purple formazan in living cells, worked well for *E. multilocularis* metacestodes in an experimental setting (Emery *et al*. 1995), but was not found to be useful for the assessment of larger numbers of compounds (A. Hemphill, unpublished observations).

Another biomarker-based approach to develop such a screening assay could be to identify specific enzyme activities in culture supernatant obtained from drug-treated parasites, which release vesicle fluid components once they are damaged or structurally impaired upon exposure to an active compound. Such biomarkers indicating damage are not present in culture supernatants of untreated metacestodes. *E. multilocularis* metacestodes are basically fluid-filled vesicles that are surrounded by an outer, acellular laminated layer, and the inner surface of this laminated layer is delineated by the actual parasite tissue, the germinal layer. The germinal layer secretes the components of the laminated layer towards the metacestode periphery into the laminated layer, and also secretes and/or releases metabolites into the vesicle fluid. One enzyme that represents an intrinsic component of the vesicle fluid, and which is also found on the laminated layer, is alkaline phosphatase (AP; Sarciron *et al*. 1991; Lawton *et al*. 1997). The detection of AP activity in medium supernatants of drug-treated metacestode cultures has been proposed as a method to screen for active drugs (Stettler *et al*. 2001). AP-activity was indeed increased in culture supernatants of metacestodes treated with nitazoxanide (Stettler *et al*. 2003), 2-methoxyestradiol and artemisinin-derivatives (Spicher *et al*. 2008*a*, *b*), but the sensitivity of this assay was not always satisfactory (Gelmedin *et al*. 2008; A. Hemphill, unpublished observations). Thus, detection of AP activity does not represent a reliable measure for drug screening *in vitro*. Another enzyme, phosphoglucose isomerase, released by dying *E. multilocularis* metacestodes, was found to be a promising marker that allows assessment of parasite viability of drug-treated *E. multilocularis* metacestodes. The potential of this screen for high-throughput assays will be published soon (B. Stadelmann *et al*. unpublished observations).

Parasite-derived biomarkers could also play a major role for monitoring drug efficacy in patients suffering from echinococcosis during drug trials. For instance, it was previously shown that the serological response of patients against *E. multilocularis* alkaline phosphatase could reflect viability following surgery and/or chemotherapy (Sarciron *et al*. 1997). Such immunological tests are dependent on the availability and specificity of antigens and do not always provide clear-cut results; the same applies to molecular tests, such as PCR, which require specific primers. Thus, more recently, disease-specific biomarkers have been identified by metabolic profiling emplyoing ^{1}H nuclear magnetic resonance spectroscopy (NMR). This technology delivers an overview of the metabolic composition of biofluids and tissues in diseased versus non-diseased individuals, and such studies have provided the means for detection and differentiation of e.g. coronary heart disease and schizophrenia (Ordovas and Moser, 2006; Holmes *et al*. 2006). Metabolic profiling has been carried out in rodents infected with *S. mansoni* (Wang *et al*. 2004), *S. japonicum* (Wang *et al*. 2006), *T. spiralis* (Martin *et al*. 2006), *Trypanosoma brucei brucei* (Wang *et al*.

Table 1. A selection of drug candidates against cystic hydatid disease caused by *Echinococcus granulosus*.

Compound	*In vitro* activity	*In vivo* activity	Activity in humans	References
Mebendazole, albendazole	Yes – cysts, protoscoleces	Yes – rodents, sheep	Yes	Rev. in Siles-Lucas and Hemphill, 2002
Oxfendazole	Not assessed	Yes – sheep, goats	Under license	Blanton *et al.* 1988; Dueger *et al.* 1999; Gavidia *et al.* 2009
Flubendazole	Yes – cysts, protoscoleces	Yes – rodents	Yes	Elissondo *et al.* 2006; 2007
Albendazole + praziquantel	Yes – protoscoleces	Not assessed	Yes	Cobo *et al.* 1998; Casado *et al.* 2001
Albendazole + ivermectin	Yes – cysts, protoscoleces	Yes – rodents	Not assessed	Moreno *et al.* 2002; Elissondo *et al.* 2009
Fenbendazole + netobimin	Not assessed	Yes – rodents	Not assessed	Garcia-Llamazarez *et al.* 1997
Cetrimide	Yes – protoscoleces	Yes – sheep, cattle (intracyst)	Not assessed	Frayha *et al.* 1981
Monensin	Yes – protoscoleces	Not assessed	Not assessed	Rogan and Richards, 1986
Levamisole	Yes – protoscoleces	Not assessed	Not assessed	Martínez *et al.* 1999
Ivermectin	Yes – cysts, protoscoleces	No – rodents Yes – sheep (intracyst)	Not assessed	Ochieng'-Mitula and Burt, 1996; Martinez *et al.* 1999; Moreno *et al.* 2002; Elissondo *et al.* 2009
Nitazoxanide	Yes – cysts, protoscoleces	No- sheep	Yes – active against progressive disease	Walker *et al.* 2004; Gavidia *et al.* 2009; Winning *et al.* 2009
Thymol	Yes -protoscoleces	No – rodents	Not assessed	Kammerer and Perez-Esandi, 1975; Elissondo *et al.* 2008
Cyclosporin A	Yes – protoscoleces	Yes – rodents	Not assessed	Hurd *et al.* 1993; Colebrook *et al.* 2004
Genistein and derivates	Yes – protoscoleces	Not assessed	Not assessed	Nagulesworan *et al.* 2006
Dihydroartemisinin, artesunate	Yes – cysts	Not assessed	Not assessed	Spicher *et al.* 2008*b*

2008), *Plasmodium berghei* (Li *et al.* 2008), and *Echinostoma caproni* (Saric *et al.* 2008), and could be of high interest for other infections where good markers for disease progression and/or regression are still missing. For instance, a recent study (Hosch *et al.* 2008) has shown that there is a good correlation between metabolic viability assessment achieved through ^{1}H NMR of *E. granulosus* cyst fluid measured *ex vivo* from 50 patients with different degrees of disease classification and the classical ultrasound/light microscopy disease assessment, demonstrating the potential benefit of the use of metabolic biomarkers in disease and parasite viability assessments, e.g. during chemotherapy.

DRUG CANDIDATES FOR THE TREATMENT OF ECHINOCOCCOSIS

Cystic echinococcosis (*CE*)

A selection of drugs and potential drug candidates against CE is provided in Table 1. In many instances, *in vitro* studies on compounds active against *E. granulosus* have employed protoscoleces. Experimental prophylactic therapy of *E. granulosus* protoscoleces was carried out as a model that would mimic spillage during surgery, by treating protoscoleces with praziquantel (Urrea-Paris *et al.* 2001) or a combination of praziquantel and albendazole (Casado *et al.* 2001) prior to injection into mice. The combination of albendazole and praziquantel has been used successfully in the treatment of human CE (Cobo *et al.* 1998). Other promising compounds with *in vitro* protoscolicidal actions against *E. granulosus* were cetrimide (Frayha *et al.* 1981) and the ionophore monensin (Rogan and Richards, 1986), but these drugs were rather ineffective against metacestodes. Levamisole and ivermectin, which are classically used against nematode infections, exhibited *in vitro* activities similar to benzimidazoles (Martinez *et al.* 1999; Elissondo *et al.* 2009). The direct injection of ivermectin into *E. granulosus* cysts in laparotomised patients has also shown parasiticidal effects (Ochieng'-Mitula and Burt, 1996). The combination of ivermectin plus albendazole has shown synergistic effects in infected mice (Moreno *et al.* 2002). Benzimidazoles vary considerably with regard to their protoscolicidal action. Albendazole and albendazole sulphoxide have been shown to be active against *E. granulosus* protoscoleces *in vitro* (Chinnery and Morris, 1986; Morris *et al.* 1987; Perez-Serrano *et al.* 1994), but the *in vitro* protoscolicidal action of these drugs is rather slow and requires a longer incubation period compared to mebendazole (Morris *et al.* 1987; Rodriguez-Caabeiro *et al.* 1989) or oxfendazole, the major fenbendazole sulphoxide metabolite. Against *E. granulosus* infection in rodents, a combination of fenbendazole and netobimin (Garcia-Llamazarez *et al.* 1997) showed synergistic effects, allowing the

administration of lower drug dosages. Oxfendazole, like albendazole, is a benzimidazole, used in veterinary medicine for the treatment of nematode infections, and has a similar antimicrobial spectrum but a longer half-life. Experimental treatments of naturally *E. granulosus*-infected sheep and goats suggested that oxfendazole may be as efficacious as albendazole, but does not require daily uptake of the drug because of its prolonged bioavailability (Blanton *et al*. 1988; Dueger *et al*. 1999). Nitazoxanide, a nitro-thiazole-analogue (Hemphill *et al*. 2006) induced severe damage to *E. granulosus* protoscoleces and the germinal layer of *E. granulosus* metacestodes within few days of *in vitro* culture (Walker *et al*. 2004). Nitazoxanide treatment was not effective against experimental *E. granulosus* infection in sheep, but oxfendazole treatment, and a combination of oxfendazole and nitazoxanide, significantly decreased the number of fertile cysts and increased the number of degenerated cysts (Gavidia *et al*. 2009). On the other hand, a recent case report has suggested beneficial effects of nitazoxanide in the treatment of refractory bony hydatid disease in a human patient (Winning *et al*. 2009). This patient had been suffering from progressive disease despite treatment with albendazole and praziquantel, and the clinical response on nitazoxanide treatment showed marked improvement in the soft tissue cysts, with stable disease in the bony pelvis. Although further studies are required, this report suggests that nitazoxanide may be an effective treatment option in CE, particularly in patients with progressive disease who are receiving conventional treatment (Winning *et al*. 2009).

Profound protoscolicidal activity was also reported for another benzimidazole-derivative, flubendazole (Ellissondo *et al*. 2006, 2009). Further studies subsequently showed that flubendazole also exhibited anti-*E. granulosus* metacestocidal activities *in vitro* and *in vivo* in experimentally infected mice (Elissondo *et al*. 2007). The same authors, together with Kammerer and Perez-Esandi (1975), showed that thymol, one of the major components of the essential oils of *Thymus*, was found to exhibit substantial protoscolicidal activity (Elissondo *et al*. 2008).

Cyclosporin A, employed mainly as an immunosuppressant during the management of organ transplants, also exhibits anti-echinococcal activity. The administration of cyclosporin A in five consecutive daily doses, beginning 2 days prior to the infection of mice with *E. granulosus* protoscoleces, resulted in a significant reduction in cyst numbers and cyst masses measured at 20 weeks after infection. However, no changes in cyst mass and numbers were recorded when the drug was administered 18 weeks after infection, but the wet weight was decreased by 42% compared with untreated controls. Ultrastructural examination of the germinal membrane and laminated layer of late-treated *E. granulosus* revealed abnormalities in all cysts studied whereas control and early-treated hydatids were normal (Hurd *et al*. 1993). Cyclosporin A has also been shown to affect *E. granulosus* protoscoleces *in vitro* (Colebrook *et al*. 2004).

Alveolar echinococcosis (*AE*)

A selection of drugs and potential drug candidates for the treatment of AE is provided in Table 2. Cyclosporin A, contrary to what was found in CE, lacked anti-parasitic activity against *E. multilocularis* infection in experimentally infected mice (Liance *et al*. 1992). Doxorubicin, or hydroxyldaunorubicin, a DNA-interacting drug used widely in the treatment of a wide range of cancers (Launchbury and Habboubi, 1993), was bound to poly-isohexylcyanoacrylate nanoparticles (a colloidal biodegradable drug carrier) and applied in *E. mutlilocularis*-infected mice, resulting in the reduction of the development of the parasite in the liver and a reduced viability of the metacestode. Free doxorubicin or unbound nanoparticles had no antiparasitic activity (Liance *et al*. 1993). Animal experimentation in rodents demonstrated parasitostatic effects of mytomicin C, piperazine and quinolone derivates, alkylaminoethers and propargylic alcohols, either at a lower level or comparable to benzimidazoles (reviewed in Siles-Lucas and Hemphill, 2002). The efficacy of praziquantel was inadequate (Marchiondo *et al*. 1994), although showing some effects on protoscoleces *in vitro* (Taylor and Morris, 1988). Also, the treatment of *E. multilocularis*-infected mice with alpha-difluoromethylornithine was not successful (Miyaji *et al*. 1993).

More recently, studies on chemotherapeutically interesting compounds have employed *in vitro* cultured parasites. Nitazoxanide, a broad-spectrum anthelminthic also used for treatment against enteric bacteria, *Giardia* and *Cryptosporidium* (Hemphill *et al*. 2006), was identified as a compound inducing significant distortion of the germinal layer *in vitro*, and nitazoxanide-treated *E. multilocularis* metacestodes were non-viable when introduced into susceptible mice (Stettler *et al*. 2003). Reuter *et al*. (2006) investigated the *in vitro* efficacy of a series of compounds against *E. multilocularis* metacestodes, including albendazole, artemether, caspofungin, itraconazole, ivermectin, methiazole, miltefosine, nitazoxanide, rifampicin and trimethoprim/sulfamethoxazole. They found that albendazole, itraconazole, methiazole and nitazoxanide effectively destroyed parasite vesicles *in vitro*. However, after drug discontinuation, re-growth of vesicles occurred, indicating a parasitostatic effect only. Combination treatment with albendazole/nitazoxanide at concentrations between 1 and 10 μg/ml for 3 weeks yielded no re-growth of parasites during 8 months of

Table 2. A selection of drug candidates against alveolar echinococcosis caused by *Echinococcus multilocularis*

Compound	*In vitro* activity	*In vivo* activity	Activity in humans	References
Cyclosporin A	Not assessed	No – rodents	Not assessed	Liance *et al*. 1992
Doxorubicin and colloidal carrier	Not assessed	Yes – rodents	Not assessed	Liance *et al*. 1993
Mitomycin C, piperazine and derivates, alkylaminoethers, propargylic alcohols	Not assessed	Yes – rodents	Not assessed	Rev. in Siles-Lucas and Hemphill, 2002
Praziquantel	Yes – protoscoleces	No – rodents	Not assessed	Taylor and Morris, 1988; Marchiondo *et al*. 1994
Mebendazole, albendazole*	Yes – metacestodes, protoscoleces	Yes – rodents	Yes	Rev. in Siles-Lucas and Hemphill, 2002
Flubendazole	Not assessed	Yes – rodents	No	Rev. in Siles-Lucas and Hemphill, 2002
Itraconazole*, methiazole*	Yes – cysts	Not assessed	Not assessed	Reuter *et al*. 2006
α-difluoromethyl-ornithine	Not assessed	No – rodents	Not assessed	Miyaji *et al*. 1993
Nitazoxanide* or nitazoxanide + albendazole	Yes – metacestodes	Yes – rodents	Only case reports. Nitazoxanide alone not effective. Some limited activity in combination with albendazole. Needs more studies	Stettler *et al*. 2003, 2004; Reuter *et al*. 2006; Kern *et al*. 2008
Artemether, caspofungin, ivermectin, miltefosine, rifampicin, trimethoprim /sulfamethoxazole	No – metacestodes	Not assessed	Not assessed	Reuter *et al*. 2006
Amphotericin B desoxycholate*	Yes – metacestodes	Not assessed	Yes (intravenous)	Reuter *et al*. 2003*a*, *b*
Genistein and derivates	Yes – metacestodes	Not assessed	Not assessed	Naguleswaran *et al*. 2006
Pyridinyl imidazoles	Yes – metacestodes	Not assessed	Not assessed	Gelmedin *et al*. 2008
2-Methoxyestradiol	Yes – metacestodes	No – rodents	Not assessed	Spicher *et al*. 2008*a*
Dihydroartemisinin, artesunate	Yes – metacestodes	No- rodents	Not assessed	Spicher *et al*. 2008*b*
Isoprinosine and derivates	Yes – protoscoleces	Yes – rodents	Not assessed	Lawton *et al*. 2001

* Parasitostatic

drug discontinuation, and the subsequent evaluation in a bioassay in gerbils did also not result in viable parasite infections. In this respect, Stettler *et al*. (2004) showed that nitazoxanide, applied orally to *E. multilocularis* infected mice, either alone or in combination with ABZ, exhibited a profound antiparasitic efficacy, with the albendazole/nitazoxanide combination yielding the most promising outcome in terms of reducing parasite weight. The pharmacokinetic analysis of corresponding serum levels in mice showed that the application of albendazole in combination with nitazoxanide increased considerably the levels and the half-life of albendazole sulfoxide (Stettler *et al*. 2004). Therefore, the increased efficacy observed in mice could be the result of an increased availability of albendazole sulfoxide in mice receiving the combination treatment. Despite these promising results, neither nitazoxanide monotherapy nor nitazoxanide-albendazole combination therapies were highly effective in human patients sufffering from AE (Kern *et al*. 2008).

Amphotericin B desoxycholate (cAMB), an antifungal compound, effectively inhibited the growth of *E. multilocularis* metacestodes, first *in vitro*, and subsequently in human patients *in vivo* (Reuter *et al*. 2003*a*, *b*). A major limitation of cAMB is its mode of administration (intra-venous), which makes it unsuitable for prolonged use, except for salvage treatment (Reuter *et al*. 2003*b*). Also, the action of cAMB is only parasitostatic and, since the drug is nephrotoxic, its widespread use is limited. Nevertheless, prolonged application of cAMB for months to years may be feasible in some cases, as side effects are mild and serious organ damage does not appear to occur (Reuter *et al*. 2003*b*).

In vitro studies on *E. multilocularis* metacestodes and *E. granulosus* protoscoleces have shown that genistein, representing a major component of soya and the most prominent isoflavonoid, as well as a number of genistein derivatives, are also highly effective against these parasites. The molecular basis of the efficacy of genistein and its derivative Rm6423 have not yet been elucidated, but these compounds could interfere in signalling, for instance, through an inhibition of the tyrosine kinase activity associated with the epidermal growth factor receptor identified in *E. multilocularis* (see Brehm *et al*. 2006). Recently, Gelmedin *et al*. (2008) identified pyridinyl imidazoles as ATP-competitive inhibitors of a p38-like mitogen-activated protein kinase (MAPK) of *E. multilocularis* by adding them to *in vitro* cultures, demonstrating death of parasite vesicles at concentrations that did not affect cultured mammalian cells.

An endogenous metabolite of oestrogen with both anti-angiogenic and anti-tumour effects, 2-methoxyestradiol (2-ME) (reviewed by Schumacher and Neuhaus, 2001) was shown to induce severe damage to *E. multilocularis* metacestodes *in vitro* in a dose-dependent manner (Spicher *et al*. 2008*a*). However, 2-ME-treatment of experimentally infected mice did not result in a reduction in parasite weight compared to the control, demonstrating that *in vitro* and *in vivo* situations are not always comparable. Best results were achieved with a treatment using a combination of 2-ME and albendazole, which lead to a reduction in parasite weight compared to albendazole treatment alone, but results did not show statistical significance (Spicher *et al*. 2008*a*). *In vitro* treatment of *E. multilocularis* and *E. granulosus* larval stages with the antimalarials dihydroartemisinin and artesunate exhibited promising results, while 6 weeks of *in vivo* treatment of mice with infected *E. multilocularis* metacestodes had no effect. Again, combination treatments of both drugs with albendazole led to a substantial but statistically not significant reduction in parasite weight compared to results with albendazole alone (Spicher *et al*. 2008*b*).

The *in vitro* effect of isoprinosine and its derivates has also been demonstrated against protoscoleces of *E. multilocularis* (Lawton *et al*. 2001).

RELEVANCE OF *ECHINOCOCCUS* METACESTODE SCREENING MODELS FOR OTHER CESTODES AND TREMATODES

Numerous studies have demonstrated that many of those drugs that were active in *E. granulosus* drug screening models were also of significant relevance for *E. multilocularis* and *vice versa* (see above). In addition, there is ample evidence that most compounds with good *in vitro* and *in vivo* efficacy against *Echinococcus* metacestodes are also of relevance for combatting infections by other cestode larval stages. For instance, cysticerci of *T. taeniaeformis* were highly sensitive to praziquantel (Becker *et al*. 1981), and the same applied to *T. solium* and *T. pisiformis* (Garcia-Dominguez *et al*. 1991; Martinez Zedillo *et al*. 1992). Albendazole and mebendazole (or modified derivatives) were also active against *T. taeniaeformis* cysticerci in experimentally infected mice (Verheyen *et al*. 1978; Jain *et al*. 1989). To date, albendazole and praziquantel, taken as short-term treatments (8–15 days) are the main drugs of choice for chemotherapeutic treatment of neurocysticercosis in humans (Garcia *et al*. 2003; Shandera and Kass, 2006). A recent *in vitro* study on *T. crassiceps* indicates that a combination of nitazoxanide and albendazole could be used for the treatment of cysticercosis infections (Palomares-Alonso *et al*. 2007), but *in vivo* evidence is still lacking. The effects of other benzimidazoles, such as flubendazole, were evaluated in *T. solium*-infected swine, with promising results (Tellez-Giron *et al*. 1981) that were subsequently confirmed in human patients (Tellez-Giron, 1984).

The activity of benzimidazole derivatives against *Hymenolepis* larvae was evaluated in the intermediate

host *Tribolium confusum*, demonstrating clear effects with regard to larval development (Novak and Blackburn, 1985). However, in the rodent model it was shown that benzimidazole drugs were only effective against *H. nana* oncosphere infection, but already developed cysticercoids were difficult to cure (Gupta *et al.* 1981; Maki and Yanagisawa, 1985), and neither flubendazole nor thiabendazole could clear *H. nana* cysticercoid infections in mice. Mebendazole and a series of modified benzimidazole derivatives were effective against *H. nana* and *H. diminuta* cysticercoids (Dubey *et al.* 1985).

In vitro studies on the effects of exposure of *Mesocestoides corti* tetrathyridia to anti-parasitic drugs revealed that liposomized praziquantel and albendazole had a deleterious effect on the parasite morphology and development (Hrckova *et al.* 1998; Britos *et al.* 2000; Saldana *et al.* 2001). The efficacy and mode of action of praziquantel were studied in the mouse model, showing that application of praziquantel had an adverse effect on the tetrathyridia burden in the liver and peritoneum (Hrckova and Velebny, 1995, 1997). Following the oral administration of mebendazole to *M. corti*-infected mice, a parasiticidal effect was observed (Heath *et al.* 1975; Eckert and Pohlenz, 1976). This is in contrast to other cestode larvae, against which mebendazole exerts a parasitostatic effect only. Deleterious actions of other drugs on *Mesocestoides* tetrathyridia, including cyclosporin A (Chappell *et al.* 1989) and albendazole (Terenina *et al.* 1998), have also been reported.

Potentially, the *Echinococcus* screening models could also have some, albeit limited, relevance for a number of trematode species, including *Schistosoma japonicum, S. manoni, Chlonorchis sinensis, Fasciola hepatica* and *Opistorchis viverrini*. For instance, praziquantel is the drug of choice for the treatment of schistosomiasis (King, 2007). The drug is highly effective against the adult worm (as for cestodes), but has only a minor activity against the larval schistosomula. Another class of drugs that show similarities between *Echinococcus* and trematodes are the artemisinins and synthetic trioxolanes. These antimalarial drugs possess a broad spectrum of activity against trematodes, causing profound damage *in vitro* and substantially reducing the worm burden in experimentally infected mice (reviewed by Keiser and Utzinger, 2007*a*, *b*). *Echinoccoccus* metacestodes were also susceptible to artesunate and dihydroartemisinin *in vitro*, while the schistosomula were found to be particularly susceptible to artemether and artesunate (Utzinger *et al.* 2002; Spicher *et al.* 2008*b*). The promising activity of mefloquine, another anti-malarial drug, in mice experimentally infected with *S. japonicum* and *S. mansoni*, has been reported (Keiser *et al.* 2009), and recent studies in our laboratory also revealed that mefloquine has a profound impact on *in vitro* cultured *E. multilocularis* metacestodes (A. Hemphill *et al.* unpublished observations).

On the other hand, albendazole and mebendazole, the main anti-echinococcal drugs, have no impact on *S. mansoni* (Schmidt, 1998). In contrast, flubendazole, a mebendazole-derivative, was active against *S. mansoni* in experimentally infected mice (Nessim *et al.* 2000; Williams *et al.* 2003), and another benzimdazole, triclabendazole, was active against *Fasciola hepatica* (Robinson *et al.* 2001). As for *Echinococcus*, *F. hepatica* was susceptible to nitazoxanide treatment *in vitro*, and clinical studies showed that nitazoxanide could be used for the treatment of fasciolasis in children and adults (reviewed in Hemphill *et al.* 2006).

WHERE TO GO FROM HERE?

As outlined in this review, considerable efforts have been undertaken to improve the therapeutic options for the treatment of CE and AE (reviewed in Vuitton, 2009). These efforts have largely concentrated on the establishment of procedures on how to manage the diseases and by setting up guidelines for treatment and classifications of disease status. Although most successful to a large extent, the current benzimidazole-based chemotherapy is far from optimal and, owing to the limited efficacy of this class of compounds, their side-effects and their costs, alternative drugs or drugs that could be integrated into a combination treatment are clearly needed. Thus, considerably more input and support is needed from academic institutions as well as pharmaceutical and biotechnological industries and governmental agencies to provide solutions for these neglected diseases. A recent survey on the financial resources going into research and development funding in 2007 has shown, that HIV/AIDS received 1·1 billion US dollars, malaria and tuberculosis obtained over 400 million US dollars each, and kinetoplastid diseases and diarrhoeal diseases were granted over 125 and 114 million dollars each. On the other hand, helminth infections as a whole, encompassing nematodes, cestodes and trematodes, received only 51·6 million dollars (Voelker, 2009).

But finances alone will not provide novel possibilities. Until recently, *Echinococcus* drug discovery has been based on rather anecdotal reports, where a limited number of drugs belonging to a certain compound class have been investigated. However, the *Echinococcus* drug discovery process has been lacking several important aspects that are compulsary for successfully identifying the best and most interesting compounds. First, we need to gain access to comprehensive compound libraries, and we need to be able to screen these libraries. This can only be done through the implementation of easy-to-handle and reliable medium-to-high-throughput *in vitro* assays that allow screening of larger numbers of

anti-parasitic drugs in an efficient manner. Preferentially, these assays do not rely on subjective microscopic evaluation, but on objective criteria, such as specific markers (e.g. enzyme activities) that indicate parasite viability/intactness or non-viability/damage, and which could be detected preferentially in an (at least) semi-automated system. The development of such assays is ongoing, but should be intensified.

Secondly, large-scale drug screening activities are only possible if sufficient numbers of parasite organisms can be generated *in vitro*. This prerequisite has been fullfilled due to the pioneering work of Brehm and co-workers (see Spiliotis and Brehm, 2008), who developed an *E. multilocularis* metacestode *in vitro* culture system that allows the generation of massive numbers of metacestodes out of a relatively small quantity of parasite tissue. This does not only enable researchers to carry out numerous drug-screening assays, but also provides the basis for biochemical studies, including the identification of drug targets for specific compounds by affinity chromatography and mass spectrometry-based sequencing.

Thirdly, it is important to have access to genomic and EST databases. In 2008, shotgun sequencing of the *E. multilocularis* genome was completed (http://www.sanger.ac.uk/Projects/Echinococcus/). This opens the door for increased use of *in silico* approaches for drug target identification, similar to the discovery of the anti-metacestodicidal activity of clarithromycin reported by Mathis *et al.* already in 2005. In addition, the availiability of a genome database permits drug target identification by affinity chromatography of parasite extracts on drug-coupled matrices and subsequent protein identification by mass spectroscopy (Müller *et al.* 2008*a*, *b*).

Furthermore, a system is required to verify putative drug targets and investigate their functional role, preferentially by genetic means, such as over-expression or silencing of genes of interest. The basis for this was set in 2008, when a method for the long-term *in vitro* cultivation and proliferation of primary cells isolated from axenically grown *E. multilocularis* metacestodes was established by Spiliotis *et al.* (2008). Isolated *E. multilocularis* cells were transiently transfected with a plasmid carrying the gene coding for the cyano-fluorescent-protein (CFP), and the corresponding gene product was expressed and detected by Western blot analysis. When co-cultured with hepatocytes, cultured *E. multilocularis* cells form aggegates, and eventually undergo complete *in vitro* regeneration of metacestode vesicles. Prospectively, this could well lead to the development of transgenic larval stages, and even adult *E. multilocularis* worms, and genetic tools can be exploited to elucidate the exact functional role of putative drug targets in different stages of development (reviewed in Brehm and Spiliotis, 2008*a*).

Finally, the *in vitro* activity of a compound that exhibits outstanding performance can be verified *in vivo*, initially preferentially in a relevant small laboratory animal model. The murine or gerbil models for primary and secondary AE (Stettler *et al.* 2004; Reuter *et al.* 2006) represent reliable tools for such *in vivo* studies.

CONCLUSIONS

Approximately 2 billion helminth infections occur in humans worldwide, and these involving the larval stages of the three cestodes *E. multilocularis*, *E. granulosus* and *T. solium* are among the most serious and life-threatening ones (Brehm *et al.* 2006). From a practical point of view, the *E. multilocularis* model, in contrast to other cestodes, clearly fulfills the criteria that would allow for intensified drug-screening processes. It displays advantages such as rapid growth and proliferation *in vitro*, access to comprehensive genomic information and EST-databases, the possibility to maintain parasite isolates routinely in laboratory mice, and to verify *in vitro* results in a relevant *in vivo* model. Currently, the monitoring of therapy effectiveness in humans is based methods based on PET scan using ^{18}F-deoxy-glucose (Reuter *et al.* 2004). After optimization, such methods could be used for the follow-up of suitable drug candidates in mice or gerbils thereby further reducing the number of animals per study. Compounds that not only act parasitostatic but also parasitocidal against *Echinococcus in vivo* have not been discovered to date, but it is conceivable that such compounds exist, and that they would be a very useful addition to the arsenal of anti-parasitic drugs against cestodes and trematodes.

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