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Review

Alveolar and cystic echinococcosis: towards novel chemotherapeutical treatment options

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Abstract

Echinococcus granulosus and *Echinococcus multilocularis* are cestode parasites, of which the metacestode (larval) stages cause the neglected diseases cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively. The benzimidazoles albendazole and mebendazole are presently used for the chemotherapeutical treatment, alone or prior to and after surgery. However, in AE these benzimidazoles do not appear to be parasiticidal *in vivo*. In addition, failures in drug treatments as well as the occurrence of side-effects have been reported, leading to discontinuation of treatment or to progressive disease. Therefore, new drugs are needed to cure AE and CE. Strategies that are currently employed in order to identify novel chemotherapeutical treatment options include *in vitro* and *in vivo* testing of broad-spectrum anti-infective drugs or drugs that interfere with unlimited proliferation of cancer cells. The fact that the genome of *E. multilocularis* has recently been sequenced has opened other avenues, such as the selection of novel drugs that interfere with the parasite signalling machinery, and the application of *in silico* approaches by employing the *Echinococcus* genome information to search for suitable targets for compounds of known mode of action.

Impact of *Echinococcus*

Echinococcosis, caused by larval stages of *Echinococcus* (Cestoda, Plathelminthes), is a life-threatening disease affecting humans and livestock. Four distinct species within the genus *Echinococcus* have been identified, i.e. *Echinococcus multilocularis*, *E. granulosus*, *E. vogeli* and *E. oligarthrus* (Thompson, 1986). At present, seven to nine species are described (Nakao *et al.*, 2007; Varcasia *et al.*, 2008). All species are potentially zoonotic, two being of significant medical importance, namely *E. multilocularis* (small fox tapeworm) as the most pathogenic, and *E. granulosus* (dog tapeworm) as the most common (Rausch, 1995; McManus *et al.*, 2003). *Echinococcus multilocularis* infection causes alveolar echinococcosis (AE) in intermediate hosts and humans, and is restricted to the northern hemisphere. The current incidence rate for Germany, 0.07/100,000 persons, is probably underestimated by a factor of 3–5 (Jorgensen *et al.*, 2008). In contrast, *E. granulosus*, the causative agent of cystic echinococcosis (CE), occurs worldwide (Schantz *et al.*, 1995) with hyperendemic areas in South America, North and East Africa, southern Europe and Central Asia (McManus *et al.*, 2003). Both, AE and CE are neglected diseases, and emergence (or re-emergence), especially in developing countries, is likely (McManus *et al.*, 2003; Eckert & Deplazes, 2004), with an increasing economic impact due to the necessity of life-long treatments (Torgerson, 2003).

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Biology of *Echinococcus*

The habitat of the adult worms is 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 take 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*. Humans represent an aberrant intermediate host that acquires disease through the accidental ingestion of eggs, with serious consequences. Eggs contain the first larval stage, the oncosphere, which actively penetrates the intestinal lining, and migrates via blood and lymphatic vessels to the target sites. Most affected organs in humans are the liver for *E. multilocularis*, and the liver, lung and other sites in the case of *E. granulosus*. There, these oncospheres develop into metacestodes representing the second larval stage and the disease-causing stage. Within these metacestodes, protoscolex development takes place in natural intermediate hosts. If an infected intermediate host is ingested by a suitable definitive host, the life cycle is concluded. Protoscolex development in humans has only rarely been described (Eckert *et al.*, 1983).

Metacestodes are fluid-filled vesicles that can be separated into two distinct structural entities, namely a cellular and an acellular compartment (see fig. 1). The outer acellular surface of the metacestode is formed by the laminated layer, a carbohydrate-rich structure synthesized by the parasite, which, in terms of thickness, is much more prominent in *E. granulosus* metacestodes (Gottstein & Hemphill, 1997). In addition, *E. granulosus* metacestodes are surrounded by a very prominent host-derived fibrous capsule, the adventitial layer composed of

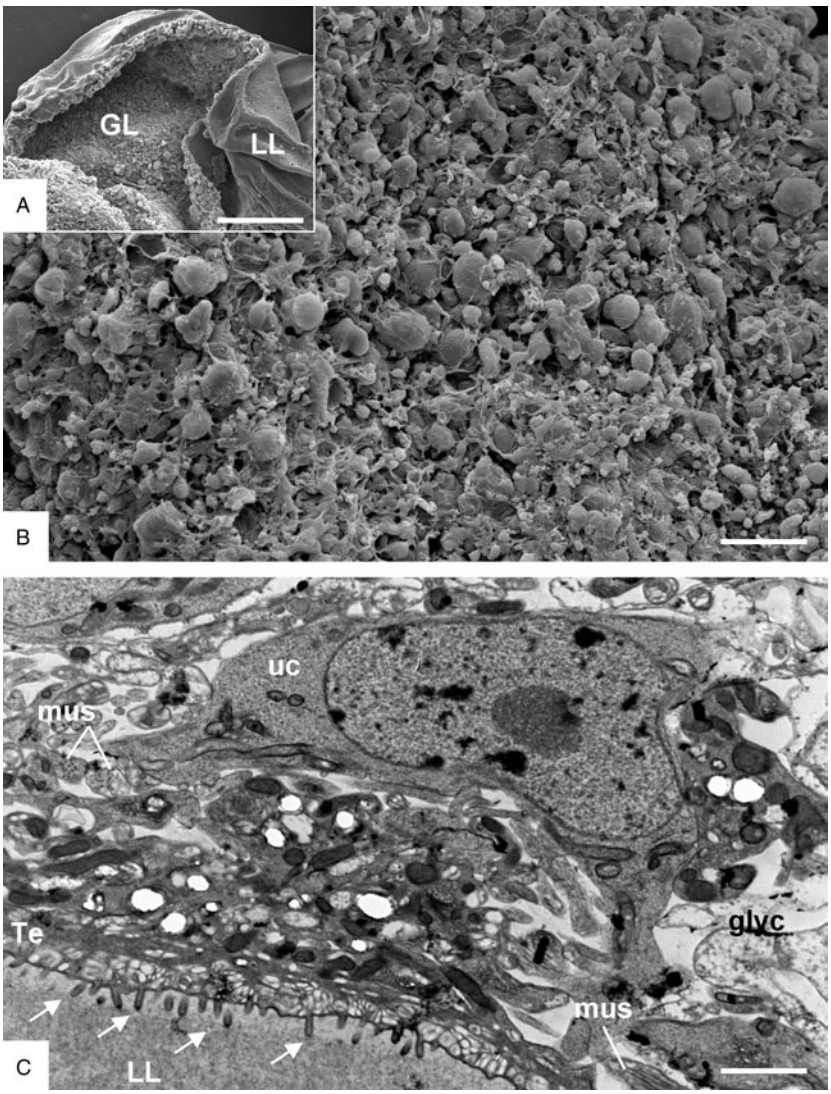

Fig. 1. Morphology and ultrastructure of *Echinococcus* metacestodes. (A) A lower magnification view of an opened metacestode exposing the inner germinal layer (GL)-associated tissue, and the acellular laminated layer representing the parasite surface (LL). Scale bar = 2.5 mm. (B) A higher magnification of the GL. Scale bar = 380 μm. (C) TEM through the metacestode wall. The parasite tissue is composed of the tegument (Te), a syncytial layer adjacent to the LL, with numerous microtriches (arrows) protruding into the LL. The germinal layer is composed of different cell types such as connective tissue cells, muscle cells (mus), glycogen storage cells (glyc), and undifferentiated cells with a large nucleus and nucleolus (uc). Scale bar = 2.5 μm.

host connective tissue. The laminated layer plays a crucial role in the survival strategy of the parasite by modulating immunological and physiological reactions on the part of the host (Gottstein *et al.*, 2002; reviewed in Siles-Lucas & Hemphill, 2002; Walker *et al.*, 2004a; Gottstein *et al.*, 2006). The actual larval tissue is formed by the germinal layer, its distal part, the tegument, being directly associated with the inner surface of the laminated layer. The tegument is characterized by microvilli-like extensions termed microtriches, which protrude well into the matrix of the laminated layer, thus increasing the resorbing surface of the parasite. 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 (Eckert *et al.*, 1983).

Typically, the fully mature *E. granulosus* metacestode (i.e. hydatid cyst) is a single-chambered or septated, but unilocular, cyst that shows expansive growth and causes thereby compression of neighbouring tissue, resulting in organ dysfunction and disease (Kern, 2003, 2006). In *E. multilocularis* infection, the metacestode represents a multi-vesicular structure that reproduces asexually, by exogenous formation and budding of daughter vesicles, which resembles progressive tumour-like growth (Ohbayashi *et al.*, 1971; Ali-Khan *et al.*, 1983). This leads to the formation of a large and heterogeneous parasitic mass consisting of mostly peripheral actively proliferating sites and, in many cases, centrally located necrotic tissue. Metastasis formation may occur in other organs due to release of germinal layer cells into the blood or lymph system (Ali-Khan *et al.*, 1983; Eckert *et al.*, 1983; Mehlhorn *et al.*, 1983). Spontaneous cure of AE leading to calcified lesions is possible, but it is not known how commonly this occurs (reviewed in Gottstein & Hemphill, 1997; Vuitton *et al.*, 2006). In fact, mass screenings in endemic areas have shown that the number of established AE infections in humans is far lower than the number of humans exposed to the eggs of the parasite and therefore sero-positive (Rausch *et al.*, 1987; Bresson-Hadni *et al.*, 1994; Bartholomot *et al.*, 2002). Thus, innate or acquired immunity is able to control the parasite after infection. This opens the door for the development of appropriate immunotherapeutical tools.

Current therapies

Traditionally, treatment of echinococcosis relies on surgery and/or chemotherapy, depending on different factors such as metacestode size and location, viability status, the interaction between the expanding parasite and the adjacent host tissue, bacterial and fungal infection, and potential complications related to cyst rupture and spillage of protoscoleces (Kern, 2003, 2006).

In CE, radical resection of the cyst mass represents the traditional treatment strategy and is, in many instances, accompanied by chemotherapy. Protoscolicidal substances are often applied since there is a risk of spilling of cyst fluid containing protoscoleces, which would be responsible for metastasis formation (Stey & Jost, 1993; Pawlowski, 1997; Kern, 2003, 2006). PAIR (puncture, aspiration, injection, re-aspiration) is a technique introduced in the mid-eighties. It includes percutaneous puncture of the cysts under ultrasonic guidance, aspiration of substantial amounts of cyst fluid, injection of protoscolicidal substance (e.g. 95% ethanol), and re-aspiration of the fluid cyst content after 15–20 min. PAIR has been used in several hundred patients. Nevertheless, the efficacy and potential risks have not been fully evaluated, and more long-term studies are needed (Morris & Richards, 1992; Brunetti *et al.*, 2004; Eckert & Deplazes, 2004).

For inoperable cases, chemotherapy with the benzimidazoles albendazole (ABZ), a broad-spectrum anthihelminthic, and mebendazole (MBZ) and the heterocyclic pyrazinoisoquinoline derivative praziquantel (PZQ) remains the only option. Pawlowski *et al.* (2001) have evaluated over 2000 well-documented inoperable cases of CE treated with benzimidazoles. When evaluated up to 12 months after initiation of chemotherapy, 30% of patients showed cyst disappearance, 50–70% exhibited cyst degeneration indicating improvement, and in 20–30% of patients *E. granulosus* metacestodes did not respond to chemotherapy (Pawlowski *et al.*, 2001). PZQ was proposed to be used alongside with benzimidazoles in CE-patients. PZQ exhibited a high efficacy against protoscoleces and metacestodes in animal experiments (Urrea-Paris *et al.*, 1999, 2001), and the combined treatment with ABZ and PZQ given during the month prior to surgery increased the number of human patients with non-viable protoscoleces, as compared to therapy with ABZ alone (Cobo *et al.*, 1998).

For the treatment of AE, surgery is always accompanied by chemotherapy using benzimidazoles, which should last for at least 2 years post-surgery, and monitoring of patients should be continued for 10 years (Pawlowski *et al.*, 2001). Inoperable AE cases must undergo long-term chemotherapy, often life-long, which is based on ABZ and/or MBZ (Reuter *et al.*, 2000, 2004). Extensive animal experimentation and observations in human patients sufferering from AE have demonstrated that ABZ and MBZ exhibit a parasitostatic rather than a parasitocidal effect (El-On, 2002; Reuter *et al.*, 2004). Therefore, 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% (Ammann & Eckert, 1995; Eckert & Deplazes, 2004).

Adverse reactions against benzimidazoles under long-term chemotherapy include elevation of transaminases, proteinuria, loss of hair, gastrointestinal disturbances, neurological symptoms (vertigo/dizziness), leucopenia, headache, abnormal liver biopsy, abdominal pain, fever, urticaria, thrombocytopenia, allergic shock (due to cyst collapse and liberation of *E. granulosus* cyst fluid) and bone marrow toxicity. A study comprised of 3282 echinococcosis patients treated with ABZ showed that most side-effects were associated with the gastrointestinal tract but no fatal cases involving ABZ therapy were described. In 3.8% of these cases, permanent discontinuation of treatment had to be undertaken (reviewed by Pawlowski, 1997; Kern, 2003). As suggested by animal experimentation, MBZ and ABZ may induce embryotoxic or teratogenic effects, and it is recommended that these drugs are not used for the

treatment of pregnant women (Horton, 1989, 1997). Constant monitoring of drug serum levels is suggested in order to avoid toxic reactions.

Taken together, besides benzimidazoles, novel drugs are required.

Methods for *in vitro* and *in vivo* evaluation of anti-echinococcal drugs

Both *in vitro* and *in vivo* laboratory models have been used for drug evaluation (reviewed in Siles-Lucas & Hemphill, 2002). Historically, the primary assessment of anti-echinococcal drug candidates has often been performed in mice or gerbils by evaluating parasite mass and/or health parameters of the host. This has led to the extensive use of animal experimentation, and has often yielded inconclusive results. *In vitro* culture of *Echinococcus* metacestodes has subsequently proven to be a suitable tool for the primary assessment of drug susceptibility by counting damaged metacestodes under the light microscope, followed by ultrastructural analysis by scanning and transmission electron microscopy (see, for example, Ingold *et al.*, 1999). A rapid test for viability is eosin exclusion (Lawton *et al.*, 2001). Another method consists in sampling culture supernatant and measuring enzymes released from damaged metacestodes (e.g. Stettler *et al.*, 2001). Furthermore, viability and proliferative capacity of metacestodes can be analysed by quantification of 14-3-3 gene expression (Matsumoto *et al.*, 2006; Spicher *et al.*, 2008a). *In vitro* cultures also represent an ideal model system for studies on drug uptake and respective metabolic changes imposed upon the parasite (Hemphill *et al.*, 2002a). *In vitro* chemotherapy studies on CE have mostly, but not exclusively, focused on protoscoleces, since these are easily cultured. Conversely, their differentiation into metacestodes is a time-consuming process and can easily take 4–6 months (Walker *et al.* 2004a, b). More recently, optimized *in vitro* culture conditions have been developed by Brehm and co-workers (Spiliotis *et al.*, 2004; reviewed by Brehm & Spiliotis, 2008). These *in vitro* methods reduce the number of animal experiments, but do not render them completely obsolete. In order to assess long-term viability and infectivity, metacestodes treated with the best drug candidates are still injected into rodents (Hemphill *et al.*, 2002).

In vitro and *in vivo* studies with benzimidazoles

Since benzimidazoles, especially ABZ, are the most relevant chemotherapeutics for the treatment of AE and CE to date, most preclinical *in vitro* and *in vivo* studies have been performed with these compounds, with a focus on comparing the activities of different benzimidazole derivatives, and on different formulations and modes of application. In rodents, efficacies of oral administration are dependent on the duration of treatment and the age of the parasite. Efficacy rises with prolongation of the treatment, but is distinctly lower for infections that have been persisting for extended periods of time (Wangoo *et al.*, 1987). Increased doses produce better results, although clear parasitocidal effects are never completely achieved (Taylor *et al.*, 1989). Moreover, phenomena related to drug resistance have been described (Morris & Taylor, 1990). Conflicting reports exist on the most suitable mode of administration of benzimidazoles. It was postulated that parenteral administration of benzimidazoles resulted in a higher efficacy than other routes in animals experimentally infected with *E. multilocularis* (reviewed in Siles-Lucas & Hemphill, 2002). Combinations of ABZ with other compounds were tested in order to obtain better treatment efficacies. For instance, synergistic effects were reported for combinations of ABZ with the dipeptide methyl ester Phe-Phe-OMe (Sarciron *et al.*, 1997). In addition, novel formulations of benzimidazoles, either as pro-drugs (Walchshofer *et al.*, 1990), liposome-entrapped compounds (Wen *et al.*, 1996) or colloidal, intravenously injectable formulations (Rodrigues *et al.*, 1995) were tested in rodents and showed enhanced efficacy at lower doses than the parental compounds. However, these studies have not really been translated into clinical applications, with one exception. Chai *et al.* (2004) reported on improved efficacy of ABZ emulsion compared to ABZ tablets or capsules for the treatment of liver CE. Experimental prophylactic therapy of *E. granulosus* protoscoleces was carried out as a model that would mimic spillage during surgery, by treating protoscoleces with PZQ (Urrea-Paris *et al.*, 2001) or a combination of PZQ and ABZ (Casado *et al.*, 2001) prior to injection into mice. Against *E. granulosus* infection in rodents, a combination of fenbendazol and netobimin (Garcia-Llanazares *et al.*, 1997) showed synergistic effects, allowing the administration of lower drug dosage. Oxfendazole, like ABZ, is a benzimidazole, used in veterinary medicine for the control 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 ABZ, but does not require daily uptake of the drug (Blanton *et al.*, 1998; Dueger *et al.*, 1999). Another benzimidazole derivative effective againt *E. multilocularis* metacestodes *in vitro* is methiazole (Reuter *et al.*, 2006).

Mode of action of benzimidazoles

When *in vitro* cultures of metacestodes were treated with ABZ, microtubular ultrastructure was affected (Rubino *et al.*, 1983; Ingold *et al.*, 1999) suggesting that ABZ interfered with tubulin polymerization (Lacey, 1990). Molecular genetics revealed that sensitivity to benzimidazoles in evolutionary distant organisms such as fungi, nematodes, platyhelmithes and various protozoa was correlated with the presence of specific alleles of β-tubulin genes (Driscoll *et al.*, 1989; Katiyar *et al.*, 1994; Kwa *et al.*, 1995; Henriquez *et al.*, 2008), the substitution of Phe to Tyr in position 200 being sufficient for switching from benomyl sensitivity to resistance in nematodes (Kwa *et al.*, 1995). Molecular modelling of the putative ABZ binding site in ABZ-resistant *Acanthamoeba* β–tubulin revealed 13 residues, four of which showed sequence variation as compared to tubulins of sensitive organisms. Resistance was conferred when, besides Phe200, Phe167 was also replaced (Henriquez *et al.*, 2008). In *E. multilocularis*, cDNAs of three β-tubulin genes

were identified, two of them having a Phe in position 200 (Brehm *et al.*, 2000). The IC_{50} values of ABZ in organisms regarded as sensitive to ABZ was between 30 nM in the protozoon *Giardia lamblia* (Katiyar *et al.*, 1994, confirmed by own observations) and more than 10 μM in nematode larvae (Satou *et al.*, 2002). Clear-cut IC_{50} values for *E. multilocularis* metacestodes are not found in the literature. Metacestodes treated with nearly 40 μM albendazole-sulphoxide (ABZSO) showed symptoms with increasing severity over time (e.g. Ingold *et al.*, 1999) and totally disintegrated only after 21 days (Reuter *et al.*, 2006). This indicates that cytotoxic effects of ABZ (or its metabolites) due to tubulin depolymerization may be hampered in *Echinococcus* by: (1) the presence of an ABZ-insensitive β-tubulin complementing the sensitive isoform; (2) rapid ABZ catabolism; (3) toxicity of ABZ catabolites rather than ABZSO itself; and/or (4) a good regenerative power due to insensitive stem cells. Sensitivity of mammalian cells (carrying the benzimidazole-resistant allele 200-Tyr) lies between an IC_{50} around 0.25 μM in HL60 cells and 20 μM in Vero cells (nocodazole, another benzimidazole, is, however, 10- to 20-fold more toxic; Katiyar *et al.*, 1994). This suggests that ABZ may also bind with low affinity to mammalian β-tubulin, and/or the existence of another, unknown, mode of action of ABZ that may be responsible for ABZ side-effects in patients, as mentioned above. In this respect, it is noteworthy to mention that Xiao *et al.* (1995) found the downregulation of enzymes involved in carbohydrate metabolism upon benzimidazole treatment of rodents bearing *E. granulosus* metacestodes.

Novel chemotherapeutical treatment options

Novel and improved therapeutical tools are needed in order to optimize treatment of CE and AE. They should have a better selectivity, thus a much greater therapeutic window than ABZ, and be parasitocidal rather than parasitostatic. Unfortunately, the pharmaceutical industry is not developing novel treatment options besides benzimidazoles against these neglected diseases. Therefore, novel chemotherapeutics have to be identified issuing from existing drugs by one of the following strategies: (1) *in vitro* testing of broad-spectrum anti-infective drugs, either in parallel with, or followed by, small animal experimentation; (2) *in vitro* testing of drugs inhibiting proliferation of cancer cells for their effects on the viability of *Echinococcus* metacestodes and protoscoleces.

Furthermore, employing the currently achieved genomic sequencing efforts, the conventional strategies (1) and (2) will be complemented by two molecular genetic and *in silico* approaches, namely (3) the exploitation of the similarities between the parasite and mammalian signalling machineries, with a special focus on targeting specific signalling receptors; and (4) the search for suitable molecular targets for compounds of known modes of action.

(1) Chemotherapeutical activities of anti-infective drugs

Besides benzimidazoles, promising compounds with *in vitro* protoscolicidal action are cetrimide (Frayha *et al.*, 1981), and the ionophore monensin (Rogan & Richards, 1986), but these drugs are rather ineffective against metacestodes. The imidazothiazole levamisole, an acetylcholine agonist, and the macrolide antibioticum ivermectin, a chloride-channel activator classically used against nematode infections, were shown to exhibit *in vitro* activities similar to benzimidazoles (Casado *et al.*, 1989; Martinez *et al.*, 1999; but see also Reuter *et al.*, 2006). In rodents infected with *E. multilocularis* metacestodes, mytomicin C, piperazine and quinolone derivates, alkylaminoethers, and propargylic alcohols exhibited parasitostatic effects, at either lower or comparable levels as benzimidazoles (reviewed in Siles-Lucas & Hemphill, 2002). PZQ has been used for the treatment of AE, but experimental data in animals have shown that the efficacy of PZQ against *E. multilocularis* metacestodes was not satisfactory (Marchiondo *et al.*, 1994). Also, treatment of *E. multilocularis*-infected mice with alpha-difluoromethylornithine was not successful (Miyaji *et al.*, 1993).

When *E. multilocularis* metacestodes were treated with the thiazolide nitazoxanide (NTZ), a broad-spectrum anthelminthic also effective against enteric bacteria, *Giardia* and *Cryptosporidium* (Hemphill *et al.*, 2006), 0.3 μM caused a total disintegration after 21 days, 3 μM after 14 days and 30 μM after 7 days at most (Reuter *et al.*, 2006). NTZ was thus more efficient *in vitro* than ABZ (see above). Ultrastructural analysis revealed that NTZ induced significant distortion of the germinal layer *in vitro* (Stettler *et al.*, 2003) and severe damage in *E. granulosus* protoscoleces and the germinal layer of *in vitro* cultured *E. granulosus* metacestodes (Walker *et al.*, 2004b). *In vitro* studies on *E. multilocularis* and *E. granulosus* employing NTZ derivatives (see Esposito *et al.*, 2007; Hemphill *et al.*, 2007) showed that metacestodicidal and protoscolicidal activity of this class of drugs depends largely on the presence of the nitro-thiazole moiety (Stadelmann & Hemphill, in preparation). *Echinococcus multilocularis* metacestodes treated with 32 μM NTZ for 14 days were non-viable when introduced into susceptible mice for 5 months (Stettler *et al.*, 2003). In another study, metacestodes treated with the same amount of NTZ were viable in gerbils (Reuter *et al.*, 2006). In contrast to ABZ, *in vivo* studies in rodents showed little or no effects of thiazolides, most likely due to their rapid metabolism. In this respect, Stettler *et al.* (2004) showed that NTZ, orally applied to *E. multilocularis*-infected mice, either alone or in combination with ABZ, exhibited a profound antiparasitic efficacy, with the ABZ/NTZ combination yielding the most promising results. Analysis of pharmacokinetics showed that the half-life of ABZ-sulphoxide, a metabolite of ABZ, increased upon application of ABZ in combination with NTZ (Stettler *et al.*, 2004). Therefore, the increased efficacy observed in mice could be a result of both the direct effects of NTZ and ABZ on the parasite and the inhibition of ABZ catabolism by NTZ. Combination treatment with ABZ/NTZ at concentrations as low as 3 and 4 μM, respectively, for 3 weeks inhibited, however, re-growth of parasites during 8 months of drug discontinuation and, also, bioassay in gerbils did not result in viable parasite infections (Reuter *et al.*, 2006). Thus, combined ABZ/NTZ treatment exhibited a parasitocidal effect. The mode of action and especially targets of NTZ in *Echinococcus* are

unknown. In *G. lamblia*, a nitroreductase was identified by affinity chromatography and subsequent enzymological studies as a potential target (Müller *et al.*, 2007b). Furthermore, recombinant protein disulphide isomerases from *G. lamblia* and *N. caninum* were inhibited by NTZ and other thiazolides (Müller *et al.*, 2007a, 2008a).

The polyene macrolide amphotericin B (AMB) is an antifungal compound binding to ergosterol in the cell membrane, thus causing membrane depolarization and cell damage by oxidative stress (Blum *et al.*, 2008). In a formulation as desoxycholate, it was shown to inhibit *E. multilocularis* larval growth *in vitro* and in human patients *in vivo* (Reuter *et al.*, 2003a, b). Major drawbacks of AMB are its intravenous application mode and its nephrotoxic side-effects. Moreover, AMB is only parasitostatic (Reuter *et al.*, 2003b). Nevertheless, a few cases of progressive human AE have been treated with AMB, and prolonged application of AMB over months to years appears to be feasible, as side-effects have been milder than expected (Reuter *et al.*, 2003b). Itraconazole, an antimycotic with a similar mode of action as AMB, was also slightly effective against metacestodes, but only parasitostatic (Reuter *et al.*, 2006).

Isoprinosine, an inosine derivative commercialized as an immunostimulant, affected viability of protoscoleces *in vitro*, thus not mediated by the immune system of the host (Lawton *et al.*, 2001).

Artemisinin and artemisinin-derivatives are widely used in malaria chemotherapy. *In vitro* testing on a number of artemisinin-derivatives showed that artesunate (AS) and dihydroartemisinin (DHA) at 40 μM caused considerable damage to metacestodes *in vitro*. However, *in vivo* treatment in mice was ineffective. Again, combination therapies of ABZ with DHA and AS, respectively, were slightly more efficacous than ABZ alone, but results closely missed statistical significance (Spicher *et al.*, 2008b).

(2) Studies on antiproliferative drugs

There are a number of links between growth of cancer cells and *Echinococcus* and other parasites (reviewed in Klinkert & Heussler, 2006), namely their proliferative capacity, the potential to modulate the immune response, the secretion of proteolytic enzymes in order to reach their target sites or organs, and the capacitiy of metastasis formation. *Echinococcus multilocularis* metacestodes behave like malignant tumours, and there is an association between the uncontrolled proliferation and growth and the overexpression in metacestodes of a family of proteins named 14-3-3 (Siles-Lucas *et al.*, 1998, 2001). 14-3-3 proteins are found in all eukaryotic cells and participate in protein kinase signalling pathways. They function as phosphoserine/phosphothreonine-binding modules and have an effect on phosphorylation-dependent events such as DNA-damage checkpoints and prevention of apoptosis (reviewed in Siles-Lucas & Gottstein, 2003). Some 14-3-3 proteins have been found to be aberrantly expressed in tumour cells, being either pro- or antitumorigenic. In fact, when *Echinococcus* 14-3-3 sequences were aligned with other 14-3-3 isoforms of other organisms, those overexpressed in metacestodes were grouped with the tumour-growth-related zeta-isoforms (Siles-Lucas *et al.*, 2001). This indicates that antitumour agents interacting with 14-3-3-triggered pathways could have the potential to interfere in growth of *Echinococcus* metacestodes.

Doxorubicin, or hydroxyldaunorubicin, is a DNA-interacting drug widely used in chemotherapy, and is commonly used in the treatment of a wide range of cancers. The parasiticidal properties of doxorubicin against the metacestode of *E. multilocularis* were investigated after binding of that drug to polyisohexylcyanoacrylate nanoparticles, a colloidal biodegradable drug carrier. A reduction of the hepatic parasite development and a reduced viability of the metacestode were observed in mice injected with 5 mg/kg body weight, but 7.5 mg/kg body weight did not appear to be more efficient. Free doxorubicin or unbound nanoparticles had no antiparasitic activity (Liance *et al.*, 1993).

Another class of antitumour agents with proven antiparasitic activities are isoflavonoids. Isoflavonoids are substances formed by plant tissue in response to physiological stimuli such as infectious agents, with reported anti-oxidant, antibacterial, antiviral and antifungal activity (Dakora & Phillips, 1996). They are composed of a characteristic 15-carbon backbone ring structure connected by a heterocyclic pyrane (3-C) bridge (C6–C3–C6) (Reynaud *et al.*, 2005), with the two aromatic rings generally containing a number of phenolic hydroxyl groups. Genistein, a major component of soya, is the most prominent isoflavonoid, and inhibits growth and metastasis of a number of cancer cell lines (breast, prostate, skin, colon). Genistein also stimulates the synthesis of transforming growth factor-β (TGF-β), which itself inhibits cancer cell proliferation (Messina, 1999). Besides other targets, genistein acts on a number of signalling pathways, by functioning as a kinase inhibitor (tyrosine kinase, mitogen activated protein (MAP) kinase, ribosomal S6 kinase). Our own studies showed that genistein was highly effective against *E. multilocularis* metacestodes *in vitro* (Naguleswaran *et al.*, 2006). However, genistein has a disadvantage, in that it also exerts oestrogenic effects by binding to oestrogen receptor-β (Pike *et al.*, 1999), and this renders genistein unfavourable for long-term treatment applications. Binding to the oestrogen receptor-β has been proven to take place through the hydroxyl-group associated with the B-ring of the molecule. We have therefore tested a number of isoflavonoids *in vitro*, which do not carry this hydroxyl-group and therefore do not meet the steric requirements to bind to the oestrogen receptor-β. One of these compounds, Rm6423, exhibits pronounced antiparasitic activity against *E. multilocularis* metacestodes, as well as against *E. granulosus* metacestodes and protoscoleces (Naguleswaran *et al.*, 2006). Further, examination of culture medium revealed increased leakage of parasite proteins into the medium during treatment, and zymography demonstrated a loss in the activity of metalloproteases. The molecular basis of the efficacy of genistein and its derivative Rm6423 have not been elucidated, but these compounds could interfere in signalling, for instance through inhibiting the tyrosine kinase activity associated with, for example, the

epidermal growth factor receptor identified in *E. multilocularis* (Spiliotis *et al.*, 2006).

2-Methoxyestradiol (2-ME), an endogenous metabolite of oestrogen with both anti-angiogenic and antitumour effects (reviewed in Schumacher & Neuhaus, 2001), was shown to downregulate the protumorigenic 14-3-3-ζ-isoform in a number of cancer cell types (Kumar *et al.*, 2003), an isoform also overexpressed in *Echinococcus* metacestodes (Siles-Lucas & Gottstein, 2003). Application of 2-ME (2–10 μM) to *E. multilocularis* metacestodes *in vitro* caused severe damage and downscaled 14-3-3 transcription levels. *In vivo* however, 2-ME had no significant effects, although a combination therapy comprised of ABZ and 2-ME produced slightly better results than ABZ alone (Spicher *et al.*, 2008a). The mechanism of action of 2-ME in cancer cells has been attributed to interference in microtubule stability and dysregulation of hypoxia-inducible factor (Attalla *et al.*, 1996; Klauber *et al.*, 1997; Mabjeesh *et al.*, 2003), inducing cancer cells to undergo apoptosis *via* extrinsic and intrinsic pathways. It is not known how 2-ME exerts its effects on *Echinococcus*.

NTZ, previously introduced as an anti-infective drug, also inhibits the proliferation of colon cancer cells *in vitro*, probably by interfering with glutathione-S-transferase (GST) class π, an isoform overexpressed in many proliferating cells (Müller *et al.*, 2008b). In *E. granulosus* and *E. multilocularis*, the only GSTs characterized so far have some sequence homologies to the mammalian class μ (Liebau *et al.*, 1996; Fernandez *et al.*, 2000). The catalytic properties of recombinant GST of *E. multilocularis* had, however, higher similarities to mammalian classes α and π, with, especially, a high conjugating activity on ethacrynic acid, an anticancer drug (Liebau *et al.*, 1996). In principle, GSTs may have two opposite effects on drugs, namely by inactivating drugs or by activating ineffective pro-drugs. The latter effects have been employed as an anticancer drug strategy (Rooseboom *et al.*, 2004) and may be further developed as an anti-*Echinococcus* strategy.

Cyclosporin A (CA) is an inhibitor of the protein phosphatase calcineurin and acts as an antiproliferative for lymphocytes, employed mainly as an immunosuppressant after organ transplantation (Ozbay *et al.*, 2007). CA also exhibits anti-echinococcal activities in mice. While administration of CA in five consecutive daily doses, beginning 2 days prior to infection with *E. granulosus* protsocoleces, resulted in significant reduction in cyst numbers and cyst masses measured at 20 weeks post-infection, no changes in cyst mass and numbers were noted when the drug was administered 18 weeks post-infection. 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). In contrast to *E. granulosus*, CA did not have any antiparasitic activity against *E. multilocularis* infection in mice, and its immunosuppressive activity was shown to be more effective than its parasitostatic effect (Liance *et al.*, 1992). Potential targets for CA in *Echinococcus* are unknown.

(3) The Echinococcus *signalling machinery as a novel drug target*

More recently, the excellent work of Brehm *et al.* has shed light on a number of developmental factors that *E. multilocularis* metacestodes share with other metazoans (for a review refer to Brehm *et al.*, 2006). These include signalling systems that employ receptor tyrosine kinases of the epidermal growth factor (EGF) (Spiliotis *et al.*, 2003, 2006), the insulin/insulin-like growth factor (Ins/IGF)-receptor families (Konrad *et al.*, 2003), and the surface serine/threonine kinases of the closely related transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP)-receptor families (Zavala-Gongora *et al.*, 2006). A cytokine that has significant homologies to mammalian EGF has been shown to be tenfold upregulated in *E. multilocularis* metacestodes cultured under conditions that promote growth and differentiation (Spiliotis *et al.*, 2003). EmSkip, a novel member of the SNW/SKIP family of transcriptional co-regulators was found to be expressed in the *Echinococcus* metacestodes and protoscoleces during an infection of the intermediate host (Gelmedin *et al.*, 2005). EmSkip interacts with EmSmadA and EmSmadB, two TGF-β/BMP signal transducers of *E. multilocularis* (Zavala-Gongora *et al.*, 2003), indicating a role of this protein in TGF-β signalling processes in the parasite. In addition, downstream signalling elements of the MAP kinase cascade have been identified and characterized (Spiliotis & Brehm, 2004; Spiliotis *et al.*, 2005, 2006). The *Echinococcus* MAP kinase cascade factors share similarities, but also have differences, compared to their mammalian counterparts. The differences would represent prime candidate targets for the development of novel anthelminthic drugs. For instance, analysis of receptor activation has shown that the *E. multilocularis* insulin receptor EmIR interacts readily with insulin from the host. Moreover, TGF-β receptor EmTR1, and possibly also the EGF-receptor EmER, interact with their corresponding host ligands (reviewed in Brehm *et al.*, 2006). Thus, parasite and host have evolved means of communication that would largely influence the developmental biology of both parasite and host. These receptor–ligand systems certainly play a central role in host–parasite interaction processes, and thus represent interesting drug targets (Brehm *et al.*, 2006). Cancer research has generated an enormous number of compounds that interfere in the functional activities of homologous receptors or respective downstream kinases (for review see Sioud & Leirdal, 2007), and the challenge will be to identify those drugs, or respective derivatives, that inhibit these receptors, or the corresponding downstream enzymes, in a parasite-specific manner.

(4) In silico *approaches*

Mathis *et al.* (2005) have been the first to exploit the current genomic sequence information to define a drug target in *Echinococcus in silico*, and subsequently confirmed their hypothesis experimentally. In bacteria, the ribosomes are important antibiotic targets, and macrolides such as erythromycin and clarithromycin are agents that bind to the nascent peptide exit tunnel near

the peptidyltransferase centre of large subunit rRNA (Rodriguez-Fonseca *et al.*, 1995). Higher eukaryotes carry a guanine at position 2058 of both cytoplasmic and mitochondrial rRNAs, and this modification at this position has been demonstrated to confer the resistance of eukaryotic cells to macrolide antibiotics. In contrast, the mitochondrial rRNA of *E. multilocularis* carries an adenine at sequence position 2058, which would be predictive for susceptibility (Sander *et al.*, 1997), while the nucleus-encoded rRNA is characterized by a guanine at 2058 (Mathis *et al.*, 2005). Upon *in vitro* culture of *E. multilocularis* metacestodes with clarithromycin, parasites, as expected, exhibited severely impaired growth characteristics, presented morphologically altered mitochondria and displayed a lack of microtriches, all in a dose-dependent manner. Adult worms were also severely affected, lost their motility and displayed morphological alterations such as shortening and constriction of proglottids and increased vacuolization. This study (Mathis *et al.*, 2005) is the first in *Echinococcus* to encourage the use of sequence-based *in silico* approaches for the exploitation of drugs, the mode of action of which is well studied at the molecular level and the corresponding target is precisely defined. However, a prerequisite for this is the availability of more comprehensive *Echinococcus* genome sequence information. In 2008, shotgun sequencing of the *E. multilocularis* genome (*c.* 270 MB) was completed (http://www.sanger.ac.uk/Projects/Echinococcus/).
This opens the door for the identification of drug targets by affinity chromatography followed by mass spectrometry (MS)-based sequencing and for reverse genetics by overexpressing or silencing genes of interest. Transient transfections of primary cells of *E. multilocularis* have already been performed (Spiliotis *et al.*, 2008).

Concluding remarks

As outlined in this review, considerable efforts have been undertaken in order to improve the therapeutical options for the treatment of CE and AE. Benzimidazole-based treatments have considerably improved the prognosis of patients, but new developments are wanted since the current treatments are only parasitostatic and have numerous side-effects. Parasitocidal compounds *in vivo* have not been identified to date, but drugs with different modes of action, such as NTZ, AMB and oestradiol- and artemisinin-derivatives, having shown promising results in preclinical studies, may be tested in combination with ABZ or related benzimidazoles in order to develop a suitable parasiticidal therapy. So far, academic institutions provide a scientific basis for novel treatment options, but financial constraints constantly limit the further development of promising therapies. Therefore, considerably more input is needed by the pharmaceutical industry and governmental agencies, in order to provide solutions for these neglected diseases. Clearly, a medium- to high-throughput metacestode screening system, allowing preliminary *in vitro* assessments of novel drug classes and respective conclusions on structure–activity relationships, needs to be developed. Besides traditional screening methods, genomic approaches, focused on the discovery of the receptor–ligand interactions and associated signalling pathways that influence the parasite–host interactions, will provide new opportunities and promising targets for follow-up studies on novel therapeutical options.

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