Drug Discovery and Development for the Treatment of Echinococcosis, Caused by the Tapeworms *Echinococcus granulosus* and *Echinococcus multilocularis*

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1 *Echinococcus* and Echinococcosis

Echinococcosis, caused by larval stages of the genus *Echinococcus* (Cestoda, Platyhelminthes), is a life-threatening disease affecting humans and livestock. The two most prominent members of the genus *Echinococcus* are *Echinococcus granulosus sensu lato* and *Echinococcus multilocularis*. The taxonomy of *E. granulosus sensu lato* is rather complex, as it includes *E. granulosus sensu stricto, Echinococcus equinus, Echinococcus ortleppi, Echinococcus canadensis, Echinococcus intermedius,* and *Echinococcus felidis* [1–4], all of which cause cystic echinococcosis (CE or hydatid disease) with different host specificities worldwide. For simplification, we collectively refer to these species as *E. granulosus. E. multilocularis* is the causative agent of alveolar echinococcosis (AE) and it occurs in the Northern Hemisphere. Other species include *Echinococcus oligarthrus*, causing unicystic echinococcosis in South America; *Echinococcus vogeli*, causing polycystic echinococcosis in South America; and *Echinococcus shiquicus* that was identified as a sister species to *E. multilocularis* more recently [2,4,5].

E. multilocularis, commonly known as the small fox tapeworm, is found to be highly endemic in regions such as Central and Western China, Russia, Western-Central Europe (classically Switzerland, Southern Germany, Eastern France, and Western Austria), Eastern Europe including the Baltic countries, and in Alaska (Northern America) and Hokkaido (Japan) [6]. Ninety-one percent of human AE cases are located in the Tibetan plateau of Western China [7]. Recently, AE has become an increasing health problem, in particular in Kyrgyzstan [6]. In Western-Central Europe, 0.3 to 3 per 1 000 000 inhabitants get infected with *E. multilocularis* annually [8]. AE also affects other species, such as dogs, monkeys, pigs, horses, beavers, and others [9].

E. granulosus (the small dog tapeworm) occurs globally, and mostly in the Mediterranean area, Central Europe, South America, Africa, and Central Asia. In addition, CE exists as an imported disease in Western Europe and in the United States [10]. In terms of case numbers and distribution, CE by far outnumbers AE; however, AE is a much more severe disease and more difficult to treat.

CE, but to some extent also AE, occurs predominantly in resource-poor communities. For AE, although also present in higher developed countries, the number of patients is most likely underestimated by a factor of 3–5 [11]. This means that investments in the development of new drugs against echinococcosis will have a low market return, and thus the pharmaceutical industry will not be compelled to develop novel drugs against echinococcosis. However, the habitats of *E. multilocularis* and *E. granulosus* have continuously expanded, and both AE and CE are now found in

regions which were previously free of disease. Thus, emergence (or reemergence), especially in developing countries, is likely, with an increasing economic impact due to the necessity of livelong treatments. The global impact of CE and AE is comparable to other neglected tropical diseases such as trypanosomiasis, Chagas disease, and schistosomiasis [12].

2 The Biological Features of *E. granulosus* and *E. multilocularis*: Similar, but Different

Both *Echinococcus* species undergo a typical predator–prey life cycle and humans represent accidental intermediate hosts. The hermaphroditic adult stage lives in the intestine of the respective definitive hosts, where it undergoes sexual development. Definitive hosts for *E. multilocularis* are the red fox and arctic fox, coyote, raccoon dog, wolf, domestic dog, and the cat [13]. The main definitive host of *E. granulosus* is the domestic dog, but other canine carnivores can also get involved. The adult worms produce infective eggs, which are released into the environment via fecal shedding. Eggs contain an oncosphere (the first larval stage) and are orally infective for a wide range of intermediate hosts. Typical intermediate hosts for *E. granulosus* are cattle, sheep, goats, pigs, and camels, while those of *E. multilocularis* are predominantly voles (such as *Microtus arvalis* or *Arvicola terrestris*), but also other small mammals, mostly small rodents, as well as dogs and sheep. Once eggs are ingested, the oncospheres get activated during the stomach passage, will be set free in the intestine, penetrate the intestinal tissue and reach blood and lymphatic vessels, and eventually reach the target organ. There it develops into a second larval stage, the metacestode. Metacestodes are characterized by continuous, potentially unlimited growth, and the differentiation into protoscoleces, namely, precursors of newly formed tapeworm heads, takes place. Once intermediate hosts or tissues containing metacestodes and protoscoleces are ingested by a carnivorous definitive host, protoscoleces evaginate and attach to the intestinal epithelium and develop into adult tapeworms, thus concluding the life cycle.

Humans represent an aberrant intermediate host for these parasites. The most affected organs in humans are the liver for *E. multilocularis,* and the liver, lung, and other sites in the case of *E. granulosus*. Metacestodes at these sites are the target of chemotherapeutical and surgical treatment approaches. Protoscolece development in humans infected with *E. multilocularis* has only rarely been described, while it is more commonly observed in individuals infected with *E. granulosus*.

Echinococcus metacestodes resemble fluid-filled vesicles, which in both species exhibit a range of common features (Figure 10.1). The wall of these vesicles is separated into an inner germinal layer representing the living and metabolically active parasite tissue, and an outer, acellular and carbohydrate-rich compartment known as laminated layer, mediating the direct physical contact with host immune and nonimmune cells [14]. In terms of thickness, the laminated layer is much more prominent in *E. granulosus* metacestodes. The distal part of the germinal layer, the tegument, is directly associated with the inner surface of the laminated layer, and is characterized by microvilli-like extensions termed microtriches. The germinal layer itself is built up by muscle cells, nerve cells (serving possibly a neuroendocrine function), glycogen storage cells, connective tissue cells, and totipotent stem cells (also called germinative cells or neoblasts) [15–17]. These stem cells make up

20–25% of all cells in the germinal layer. They are responsible for the high regenerative potential of the parasite, and they are thought to be responsible for metastasis formation [18–20]. The germinal layer secretes vesicle fluid into the interior of the metacestodes, and vesicle fluid plays a role in nutrition and in exchange of metabolites within the parasite. For *E. granulosus*, the vesicle fluid is also termed hydatid fluid. *E. granulosus* metacestodes are, in addition, surrounded by a prominent host-derived adventitial layer, which is largely composed of collagenous fibers.

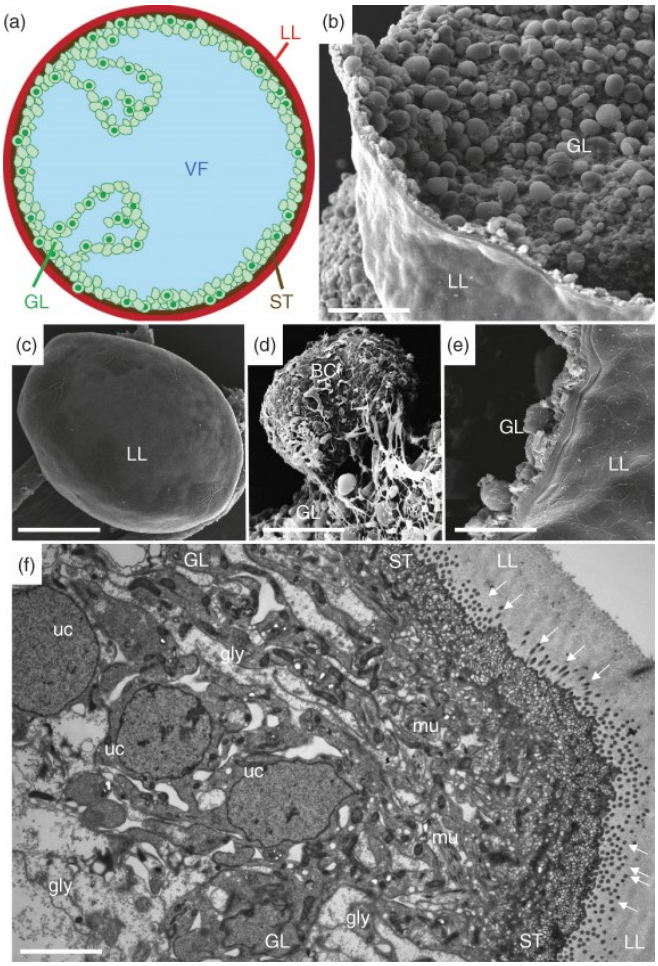

Figure 10.1 Structure of *E. multilocularis* metacestodes. Part (a) depicts a schematical view of a metacestode vesicle. The main components are indicated as color-coded: the laminated layer (LL, red); the syncytial tegument (ST, brown); the germinal layer (GL, green), the vesicle fluid (VF, blue). Parts (b)—(e) are scanning electron micrographs (SEMs) of *E. multilocularis* metacestodes. Part (b) allows a view into the interior of a metacestode, showing the germinal layer (GL) and the outer laminated layer (LL). Part (c) is an intact metacestode, with only the LL exposed, and (d) is a developing brood capsule (BC) still attached to the germinal layer (GL). A higher magnification SEM image of the vesicle wall is shown in (e). Part (f) is a section that was cut through the vesicle wall, shown by transmission electron microscopy (TEM). Note the outer laminated layer (LL), the syncytial tegument (ST) with microtriches protruding outwards into the LL (arrows), and the complex germinal layer (GL), containing undifferentiated cells (uc), muscle cells (mu), glycogen storing cells (gly), and also connective tissue. Bars in b = 330 µm; c = 1200 µm; d = 360 µm; e = 280 µm; f = 4.1 µm. (From Ref [166].)

E. granulosus metacestodes grow by expansion rather than by proliferation. Multiplication takes place internally, resulting in septated, multichambered cysts. The entire parasite mass progressively grows in size and compresses the neighboring tissues. Metastasis formation can occur upon rupture of such cysts and leakage of hydatid fluid containing protoscoleces, which themselves can then differentiate into new metacestodes in peripheral tissues. In contrast, *E. multilocularis* metacestodes represent multivesicular organisms that reproduce asexually, by exogenous formation, and budding of daughter vesicles. This process is often referred to as "progressive infiltrative tumor-like growth," and leads to the formation of a large and heterogeneous parasitic mass. This mass consists mostly of peripheral, actively proliferating, sites, and, in many cases, centrally located necrotic tissue, all intermingled with host connective tissue. Metastasis formation can occur in neighboring organs such as the gall bladder, abdominal lymph nodes, pancreas, diaphragm, and peritoneum, and in more distant regions (lungs, bones, brain, etc.) and lead to severe complications in treatment [7].

3 Clinical Hallmarks, Diagnosis, and Prevention and Control of CE and AE

The clinical presentation of CE is highly variable, and is dependent on several features such as the involved organ and the location of the cyst within the organ, cyst dimension, and the interaction with surrounding structures. In addition, different genotypes exhibit different growth characteristics, which will also impact on the clinical features [14]. Most patients (up to 80% of all cases) have a single cystic lesion located in a single organ. The liver is affected in 70% of the cases, the right lobe more commonly than the left, followed by the lungs in about 20% of the cases. Cysts can localize in any other organ and structure, such as abdominal or pleural cavities, kidney, spleen, bone, brain, vertebral column, ovary, breast, axillary region, and heart [21]. Rare immune-mediated reactions such as urticaria, asthma, membranous nephropathy, and anaphylaxis have also been well described [14]. Clinical signs may occur after a highly variable incubation period of several months or years, but hepatic cysts may remain asymptomatic for periods of up to 10–12 years. They can cause pain in the upper abdominal region, cholestasis, hepatomegaly, biliary cirrhosis, portal hypertension, ascites, and a variety of other manifestations [14]. Infection with *E. multilocularis ,* affecting the liver in the vast majority of cases, is largely asymptomatic and remains mostly undiagnosed until a progressive state is reached. This time span can reach 10–15 years. At this stage, nonspecific symptoms such as abdominal pain, jaundice, cholestasis, hepatomegaly, fever, anemia, weight loss, and pleural pain can occur [7,10].

For both AE and CE, it is, in many instances, noninvasive imaging techniques such as ultrasound, computed tomography, or magnetic resonance imaging that give the first, often coincidental, morphological indications of the disease. Ultrasound examination is the most widely used technique for screening purposes or examining individuals, and also for monitoring of postoperative and peri-therapeutic cyst development [22]. For screening purposes and confirmation of imaging findings, immunodiagnosis (ELISA and/or Western blot) is performed on the basis of a variety of native and/or recombinant antigens [23]. Biopsy retrieval and direct detection of parasite DNA by PCR or of parasite tissue by histology/immunohistochemistry is less common due to the risk of metastases formation, but can be used for confirming the diagnosis postoperatively [23]. For AE, information on the viability status of parasite lesions can be gained by F18-fluorodeoxyglucose-positron emission

tomography (FDG-PET), which highlights peri-parasitic inflammation as a response to the parasite lesion, contrast-enhanced ultrasound (CEUS), and serological follow-up or RT-PCR [24–26].

4 Currently Applied Benzimidazole Treatments for CE and AE

Surgery and chemotherapy based on the benzimidazole carbamate derivatives albendazole (ABZ, **1**) and mebendazole (MBZ, **2**) (Figure 10.2) are the two treatment options for CE and AE that are currently applied. For CE, surgery was considered the gold standard treatment until the 1980s. However, invasive surgery may be impractical, or even not feasible, in many cases, such as in patients with multiple cysts in several organs, or in patients living in regions that lack adequate facilities for advanced surgery [27]. PAIR (puncture, aspiration, injection, reaspiration) can be considered as an alternative to conventional surgery [28,29]. PAIR is minimally invasive, and includes (i) percutaneous puncture of the cyst under ultrasonographic guidance, (ii) aspiration of the cyst fluid, (iii) injection of a parasiticidal solution, and (iv) reaspiration of the fluid content after five minutes. Although hypertonic NaCl solution (20% final concentration in the cyst fluid) is not optimally effective, it is currently the most-used scolicidal solution in PAIR, as its application induces the lowest adverse side effects.

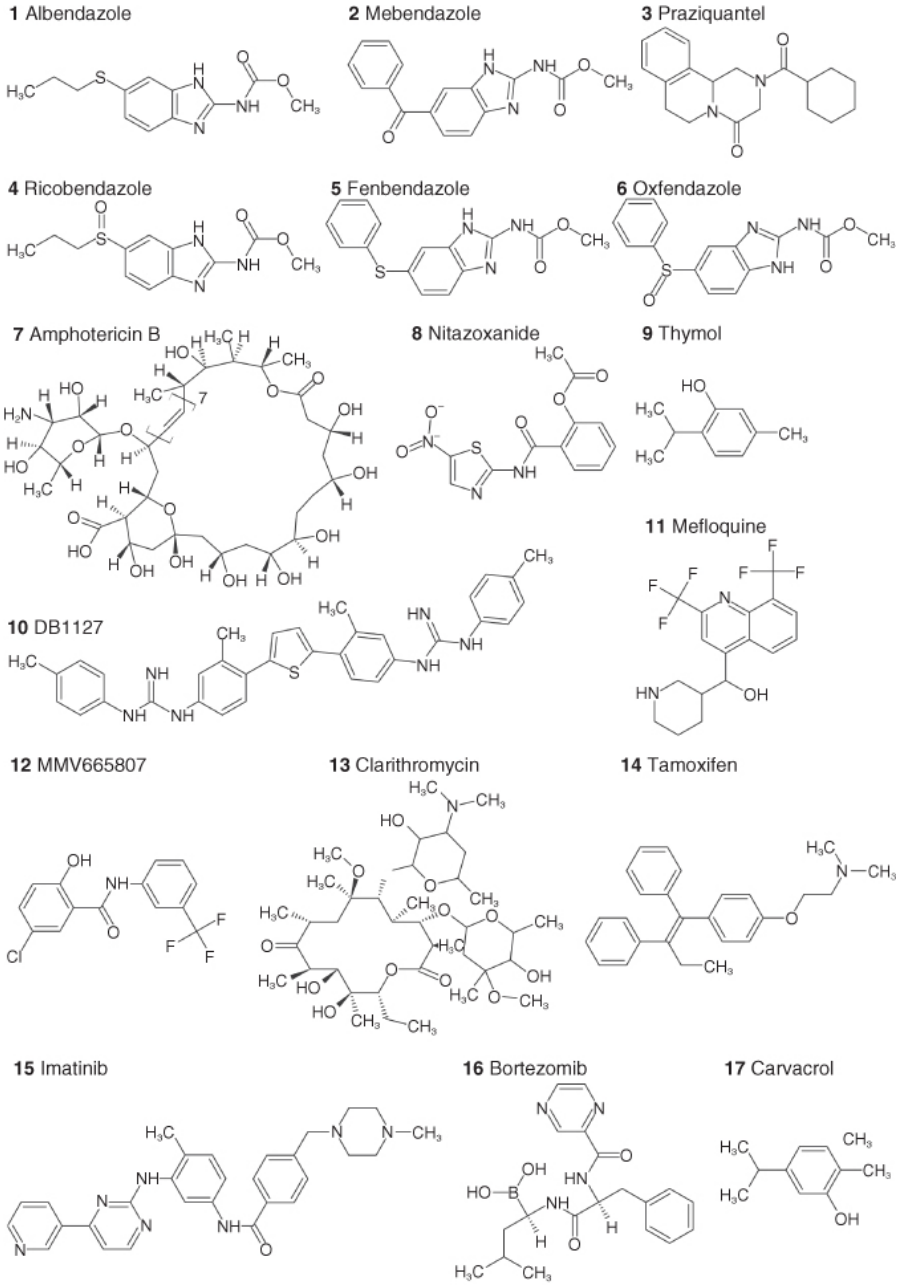

Figure 10.2 Chemical structures of selected compounds with activity against *Echinococcus*. Structures were prepared in ACD/ChemSketch 2017.1.2.

For inoperable cases, chemotherapy with the benzimidazoles ABZ and MBZ remains the only option. In the case of CE, a combined therapy using ABZ and the heterocyclic pyrazinoisoquinoline derivative praziquantel (PZQ, **3**) (Figure 10.2) has been suggested. PZQ exhibited promising efficacy against protoscoleces and metacestodes in animal experiments [30,31] and was proposed to be applied during the month prior to surgery alongside with ABZ, since this increased the number of human patients with nonviable protoscoleces as compared to therapy with ABZ alone [32,33]. Benzimidazoles must be taken over extended periods of time, often lifelong. However, under long-term benzimidazole therapy, adverse reactions such as hepatotoxicity may occur. Adverse effects can be avoided by introducing regular monitoring of drug serum levels and, if necessary, adjustment of the dosage, but this is highly dependent on a health service with a functional infrastructure, which does not exist in many countries. In general terms, benzimidazole therapy has significantly improved the life

expectancy and the quality of life of many affected patients. A study carried out with 3282 patients with echinococcosis treated with ABZ showed that gastrointestinal tract problems represent the most common adverse events, but no fatal cases were described [34]. MBZ and ABZ may induce embryotoxic or teratogenic effects [35]. Of the over 2000 well-documented inoperable cases of CE treated with benzimidazoles evaluated up to 12 months after initiation of chemotherapy, 30% of patients showed cyst disappearance, 50–70% exhibited cyst degeneration, and in 20–30% of patients *E. granulosus* metacestodes did not respond to chemotherapy [36].

Unfortunately, the prognosis is less favorable for patients with AE. The only curative treatment is invasive surgical resection of the entire parasite lesion including a safety margin, and this is applied in 20–50% of all cases [7,28]. However, radical surgery can often not be performed as most cases are diagnosed at a late stage and the parasite grows highly invasive. According to a long-term cohort study in Germany, complete surgery could only be performed in 16.1% and 36.1% of all patients with AE (referring to cases described before and after the year 2000, respectively) [37]. If surgery is carried out, it is always accompanied by benzimidazole chemotherapy, for at least two years thereafter, and monitoring of patients should be continued for 10 years [33]. Inoperable cases of AE must undergo long-term/mostly lifelong MBZ or ABZ chemotherapy. Nevertheless, clinical studies have shown that chemotherapy has significantly increased the 10-year survival rate of inoperable or non-radically operated patients with AE from 6–25% to 80–85% [10,37]. A major setback of the current benzimidazole therapy is that ABZ and MBZ exhibit a parasitostatic rather than a parasiticidal effect *in vivo* [38]. Therefore, recurrence rates after treatment interruption are relatively high, especially in those patients not followed up with appropriate prognostic tools [26,38]. Disease progression due to treatment failure was described in up to 16% of all AE cases [39]. In countries with well-developed health care systems, where access to treatment and drug level monitoring is secured, an improved clinical management of AE can be achieved. However, the costs for treating one patient with AE amount to 108 762 Euros annually [40]. Thus, AE is still a lethal disease in less-developed countries with low, or no, financial resources [7]. A long-term study carried out in Germany showed that 54.5% of all patients experienced mild side effects, and 6.9% of the patients experienced life-threatening adverse effects such as hepatotoxicity that led to treatment discontinuation [37]. With increasing numbers of patients and no alternative to benzimidazoles developed so far, new and better treatment options are urgently needed.

One possible explanation for the parasitostatic, but not parasiticidal, effects of ABZ can be found at the molecular level. Upon oral uptake, ABZ is rapidly converted into ABZ-sulfoxide (also called ricobendazole, **4**; Figure 10.2), and at a later stage ABZ-sulfoxide is metabolized to ABZ-sulfone. The major metabolite ABZ-sulfoxide is known to bind to a distinct site on β-tubulin subunit of the tubulin dimer, and thus interferes in the polymerization of microtubules, thereby blocking many cellular functions and impairing uptake of nutrients and parasite growth [41]. In the *E. multilocularis* genome, there are three β-tubulin genes, *tub1, tub2,* and *tub3*. The stem cells that develop in the germinal layer of *E. multilocularis* express mainly the Tub-2 isoform. The ABZ-sulfoxide binding site on the Tub-2 protein is altered, and does not bind to ABZ-sulfoxide; thus, Tub-2 is resistant to the dosages of benzimidazoles used in standard treatment [42]. Other factors such as the limited half-life and uptake of benzimidazoles by the parasite could also account for the failure in parasite killing. In addition to

beta-tubulin, several metabolic enzymes were described as targets of benzimidazoles [43], including the fumarate reductase system, which constitutes the malate dismutation pathway in many helminths including *Echinococcus* [44,45], but these findings have not been further followed up. Electron microscopical studies have shown that benzimidazole treatment of *E. multilocularis* metacestodes has a rapid effect on the structural integrity of tegumental microtriches. This was shown not only for ABZ and its metabolites [46] but also for fenbendazole (**5**) and oxfendazole (fenbendazole-sulfoxide, **6**; see also Figure 10.2). However, microtriches do not contain any microtubules [47]. Therefore, additional targets of benzimidazoles in *Echinococcus* remain to be identified. A very recent study has shown that ABZ treatment increases the host immune response against the parasite [48]. To what extent this has an impact on the efficacy of the drug is not clear, but the crosstalk between chemotherapy and immunity should be further investigated.

Besides benzimidazoles, only two other compounds have reached clinical application against AE or CE. The antifungal agent amphotericin B (**7**, Figure 10.2) was applied as a salvage treatment, but it did not exert parasiticidal activity, and induced nephrotoxicity under long-term usage [49]. Nitazoxanide (**8**, Figure 10.2), a broad-spectrum anti-infective thiazolide, did also not fulfill the hopes that were put into that compound: despite promising activities in mouse studies, nitazoxanide failed to be active against human AE [49,50]. However, a few studies suggested that nitazoxanide may be an effective treatment option in CE, particularly in patients with progressive disease who are receiving conventional therapy [51–53].

5 *In vitro* and *in vivo* Models to Study Drug Efficacy and Drug Targets in *Echinococcus*

The well-established, easy-to-handle, and standardized *in vitro* culture of the *E. multilocularis* metacestode stage [54], the public availability of its genome sequence and transcriptome information [55], established stem cell culture, and the development of methods for genetic manipulation [56] have rendered *E. multilocularis* the prime model for the study of diseases inflicted by cestodes in humans. This includes not only AE but also CE as well as cysticercosis, caused by metacestodes (cysticerci) of the closely related *Taenia solium*. The genome and transcriptome of the closely related *E. granulosus* has also been published [57–59], and comparative genomics has revealed surprisingly little differences in genome structure and content between *E. granulosus* and *E. multilocularis*.

In vitro culture of *E. multilocularis* metacestodes has been reported as early as 1957 [60]. Other methods developed later [61,62] did not result in efficient production of metacestodes that would allow large-scale *in vitro* drug efficacy studies. In addition, the earlier drug studies relied solely on morphological observations rather than on objective assays for viability assessment (reviewed in Hemphill et al. [63]; [33]). In 2004, Spiliotis and Brehm published a revolutionary culture method that allows generating large amounts of metacestodes in coculture with hepatoma feeder cells [54]. This provided the basis for long-term culture and proliferation of metacestodes, axenic metacestode culture without feeder cells, and also allowed to isolate the cells of the germinal layer (“primary cells”), of which up to 82% were stem cells, which will, upon *in vitro* culture, again form infective metacestodes

[19,54,64]. Based on this metacestode and stem cell culture techniques, Stadelmann et al. established a screening cascade (Figure 10.3) that allows medium-throughput drug screening based on objective criteria [65]. As a first step, the physical impairment of *E. multilocularis* metacestodes is assessed quantitatively by measuring phosphoglucose isomerase (PGI) in the culture supernatant. This enzyme is an abundant vesicle fluid component, which is released upon physical impairment of the metacestode vesicle due to drug treatment [66]. This quantitative assay allows determination of EC_{50} values and analyses of structure–activity relationships of tested compounds. Similar approaches were also applied for related species such as *T. solium* or *E. granulosus* [67,68]. Cytotoxicity of the same drugs to mammalian cells is measured by conventional alamar blue assay to explore a potential therapeutic window. The effects of selected compounds on germinal layer cell (and stem cell) viability can be assessed by measuring ATP production by commercially available kits [65]. Finally, a test based on alamar blue assay that measures the viability of germinal layer cells within intact metacestodes (metacestode viability assay) was developed [65].

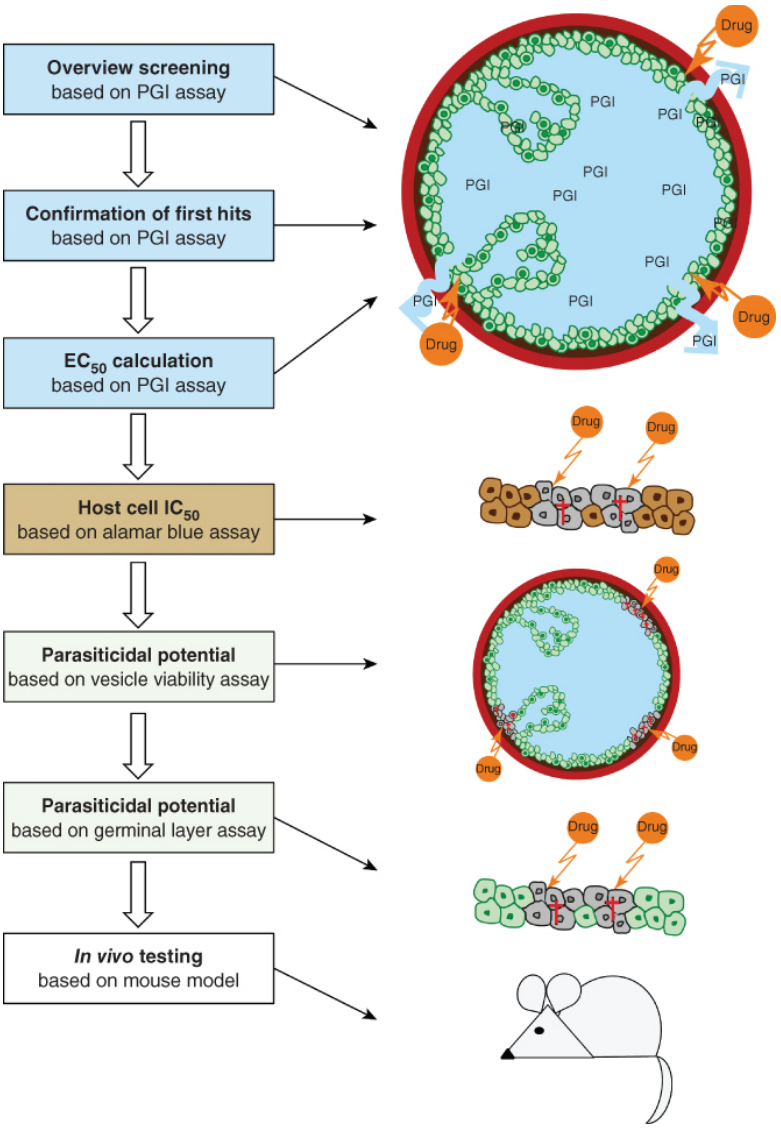

Figure 10.3 *In vitro* screening cascade of compounds against *E. multilocularis*. The three first steps of the screening are based on the PGI assay that detects metacestode damage. It is followed by host cell toxicity assessments; and only if a potential therapeutic window can be seen, further tests on potential

parasiticidal activity are included. Parasite cells are depicted in green, host cells in brown, dead cells in gray, and drugs in orange. (From Ref [166].)

For the most promising compounds, morphological effects are frequently visualized by electron microscopy, which can already indicate a potential mode of action or target organelle. Molecular targets can be identified by pull-down studies of immobilized drugs on a matrix, through which parasite extract is passed [69], or by comparing the changes in the transcriptome or proteome of parasites treated with specific drugs. Also, metabolomic studies based on NMR were applied in the past to get further insights into the mode of action of drugs [46]. Reverse genetic approaches based on RNAi are applied to validate targets of interest in protoscoleces and stem cells [70,71].

Once a compound with promising *in vitro* efficacy and selective toxicity is identified, it can be evaluated *in vivo*. Mice, as the natural intermediate host of *E. multilocularis*, represent an ideal experimental model. Two infection models can be distinguished: (i) a secondary infection model, in which mice are either intraperitoneally [72] or subcutaneously [73] infected with *E. multilocularis* vesicle suspension; (ii) a primary egg infection model [74], in which mice are orally infected by applying *E. multilocularis* eggs by gavage, thus representing the natural infection mode (Figure 10.4). In both models, treatments with drugs are initiated four to six weeks postinfection, and can last up to eight weeks or even longer, depending on compound properties and application mode. Ideally, compounds are applied orally, since multiple injections raise ethical concerns with regard to animal experimentation. Critical issues are compound formulation, mode of administration, frequency, and dosage. The formulation of drugs in honey was proposed by Küster et al. to make laboratory mice voluntarily ingest the compounds [75], but this approach is not feasible for all compounds (own observations). The *in vivo* mouse model for drug testing has been improved and standardized over the past years [73,76,77]. At the end of treatment, the final parasite mass is assessed upon necropsy and compared to placebo-treated animals. Determination of the parasite weight is the most accurate method of choice and gives a clear readout of *in vivo* drug efficacy against AE [76].

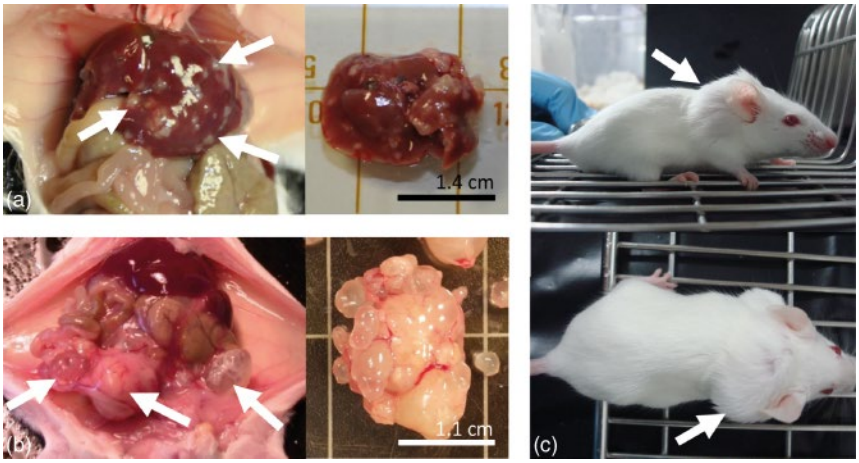

Figure 10.4 Different *in vivo* models for AE in mice. Peroral infection with *E. multilocularis* eggs resulting in liver lesions (a), intraperitoneal infection with metacestode material resulting in peritoneal lesions (b) and subcutaneous lesions visible from the outside (c, from [73]). Growing parasites are indicated by arrows.

For *E. granulosus*, respective *in vitro* culture of the metacestode stage is achieved by either culture of protoscoleces for several months until they differentiate into small metacestodes or the

metacestodes are removed from the peritoneal cavity of an experimentally infected rodent and maintained *in vitro* without proliferation. Until very recently, the only assays to assess viability of protoscoleces were based on microscopical inspection of specimens subjected to a dye (e.g. eosin, trypan blue) that stains nonviable, but not viable, parasites, and corresponding assessments are not purely objective. A newly developed technique, based on an automated assessment of drug effects by monitoring of protoscolex movement, was introduced by Ritler et al. [78]. This assay was developed for *E. multilocularis,* but is surely also applicable to *E. granulosus* protoscoleces, and allows an improved objective and higher throughput assessment of drug effects compared to microscopical inspection. To investigate the impact of drugs on the viability of *E. granulosus* metacestodes *in vitro*, trypan blue incorporation into the detached germinal layer has also been assessed in most publications, but this method is rather subjective; and for the future, more quantitative assays such as the PGI assay [66] or the motility assay [78] should be employed. The time span between infection of rodents with *E. granulosus* protoscoleces and metacestode development can take 6–10 months, as opposed to 2–3 months for rodents secondarily infected with *E. multilocularis* metacestodes. Although experimental chemotherapy with *E. granulosus* is more time-consuming, numerous interesting studies have been carried out. In many instances, *in vitro* studies on compounds active against *E. granulosus* have employed protoscoleces, since these can be obtained in a local abattoir, and it was shown that several compounds that exhibit protoscolicidal activities are not active against the metacestode stage [63]. Conversely, compounds that exhibit profound activities against *E. multilocularis* metacestodes are most likely also efficacious against *E. granulosus* metacestodes [63].

6 Drug Repurposing as the Only Strategy for Discovering Novel Compounds to Treat Echinococcosis

Realistically, it is unlikely that the pharmaceutical industry will invest in the development of a novel compound for the treatment of echinococcosis, since these investments will not result in a relevant market return. In addition, echinococcosis is a disease that is difficult to diagnose, and rather time-consuming to treat, and monitoring of treatment success is a complex undertaking, since success or failure will become evident only after months or several years of treatment. Thus, echinococcosis is also not high on the list of those foundations and private public partnership organizations that are devoted to providing better health care for neglected tropical diseases, but at the same time want (or are obliged) to deliver success stories in a timely manner. Thus, additional compounds with promising anti-*Echinococcus* activities can only be identified and implemented by exploiting the plethora of drugs that are currently on the market or being developed for other indications. Therefore, drug screening efforts have so far largely focused on already existing drugs or compound classes from other research areas. These include mostly broad-spectrum anti-infective drugs, and drugs that inhibit cellular proliferation such as anticancer compounds, but also natural products. In most studies, however, only relatively small numbers of compounds could be evaluated within a given time frame. This still stands true for *E. granulosus* (see Section 10.5); but for *E. multilocularis,* with the establishment of

optimized *in vitro* culture approaches and a standardized screening cascade, medium-sized drug libraries could be screened.

6.1 Drug Repurposing for the Discovery of Novel Compounds to Treat AE

The search for novel treatment options against AE has focused on two major areas: (i) anti-infective drugs, and (ii) anticancer drugs. The first area is obvious: *E. multilocularis* metacestodes exhibit invasive growth, are highly adapted to a parasitic lifestyle, and exert considerable immunomodulation. These features are comparable to other pathogens, and therefore drugs that affect other pathogens could also be efficacious against *E. multilocularis*. The focus on anticancer drugs can be explained by the fact that *E. multilocularis* metacestodes and malignant tumors share some distinct features: they have an unlimited proliferative capacity, express similar cell cycle regulators such as 14-3-3 protein, modulate the immune response, secrete proteolytic enzymes to reach their target sites or organs, induce angiogenesis, and exhibit the capacity to form metastases. These similarities suggested early on that antiproliferative compounds could also affect *E. multilocularis* metacestodes. In addition, it is not surprising that many compounds that exhibit broad-spectrum anti-infective activities (including artemisinins, mefloquine, nitazoxanide-derivatives and benzimidazoles) show clearly elevated toxicity in proliferating cells [69,79].

6.1.1 Anti-Infective Agents

Earlier animal experimentation studies in rodents demonstrated parasitostatic effects against experimental echincoccosis of mytomicin C, piperazine, quinacrine hydrochloride, chloroquine phosphate, alkylaminoethers, and propargylic alcohols, either at a lower level or comparable to benzimidazoles (reviewed by Siles-Lucas and Hemphill [72]). *In vivo* treatments with praziquantel were shown to enhance growth of the parasite rather than diminish it [80], and therapy employing α-difluoromethylornithine against secondary AE was ineffective [81]. The impact of other anti-infective compounds was studied *in vitro,* including ABZ and its metabolites ABZ-sulfoxide and ABZ-sulfone [46]. Conversely to what was previously thought, ABZ-sulfone exhibited anti-metacestode activity similar to that of ABZ and its sulfoxide. Other anti-infective agents such as nitazoxanide [82], artemether, caspofungin, itraconazole, ivermectin, methiazole, miltefosine, rifampicin, and trimethoprim/sulfamethoxazole were assessed *in vitro* [83]. ABZ and its two metabolites along with itraconazole, methiazole, and nitazoxanide effectively destroyed parasite vesicles *in vitro*, but regrowth of vesicles was noted once these compounds were removed. Thus, only parasitostatic effects were demonstrated. Fenbendazole was equally active as was ABZ when applied in experimentally infected mice [47]. This is not surprising, since benzimidazoles appear to act with an identical mode of action on microtubules, and thus inhibit a variety of cellular functions related to the integrity of the cytoskeleton. In support of this notion, molecular genetics revealed that sensitivity to benzimidazoles in evolutionary distant organisms such as fungi, nematodes, platyhelminthes, and various protozoa was correlated with the presence of specific alleles of β-tubulin genes (reviewed in Hemphill and Müller [84]). Like other benzimidazoles, fenbendazole and its metabolites are believed to interfere with microtubule formation by binding to free β-tubulin of the parasite, thus interfering with microtubule-

dependent uptake of glucose [41]. Improved *in vivo* efficacy could probably be achieved by employing the prodrug febantel, which is much better absorbed, and this would result in a prolonged half-life of the active oxfendazole. *In vivo*, febantel undergoes cyclization to fenbendazole, which is interconvertible with oxfendazole [47].

Nitazoxanide is a broad-spectrum antiparasitic drug belonging to the thiazolide family, with reported antiparasitic, antibacterial, and antiviral activities [85]. Besides being effective against *E. multilocularis* metacestodes and *E. granulosus* protoscoleces and metacestodes *in vitro* [52,82], nitazoxanide, applied orally by gavage, was also effective in experimentally infected mice against CE and AE. Furthermore, an ABZ-nitazoxanide combination treatment was shown to be more effective than ABZ alone [74]. However, this effect was not caused by a synergistic mode of action, but both compounds are most likely metabolized by the same Cyp450 enzyme, resulting in a delay of metabolic ABZ-sulfoxide conversion to ABZ-sulfone due to the competition of nitazoxanide and causing a prolonged presence of ABZ-sulfoxide in the serum [74]. In human patients with AE, neither nitazoxanide monotherapy nor ABZ-nitazoxanide combination therapies were effective [49,50]. Besides nitazoxanide, 29 nitazoxanide derivatives were assessed for anti-*E. multilocularis* metacestode activity *in vitro* using the PGI assay [66]. Nitro compounds, similar to nitazoxanide, but also halogenated molecules with halogenations on the thiazole moiety and also on the salicyl moiety of the thiazole scaffold exhibited enhanced PGI activity values, and extensive morphological damage was noted already after five days of treatment [66]. Thus, thiazolides are a promising class of compounds. Unfortunately, Romark LC (www.romark.com), which brought nitazoxanide on the market, was not willing to invest in a neglected tropical disease that would not bring substantial market return, and the development of thiazolides for treatment of AE was halted.

Another compound showing promising efficacy against murine AE in combination with ABZ is thymol (**9**, Figure 10.2). Thymol and a combination of thymol with ABZ exhibited promising efficacy against protoscoleces and metacestodes *in vitro* [86]. In experimentally secondary infected mice, combined ABZ/thymol treatment for 20 days starting at seven weeks after infection resulted in a considerably reduced parasite weight compared to ABZ or thymol treatments alone [87]. However, we have not been able to confirm these *in vitro* efficacy results in our own laboratory employing the *E. multilocularis* protoscolex movement assay [78] and PGI assay [66] (own unpublished findings).

The antifungal compound amphotericin B desoxycholate (cAMB) was shown to inhibit the growth of *E. multilocularis* metacestodes *in vitro*, and in human patients *in vivo* [88,89]. A major limitation of cAMB is the intravenous application mode, which makes it unsuitable for prolonged use, except for salvage treatment. Also, the effect of cAMB is only parasitostatic and the drug is nephrotoxic. 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 [89].

Earlier studies had shown that pentamidine, belonging to the class of di-cationic compounds developed against intracellular and extracellular protozoan parasites such as *Leishmania*, *Trypanosoma*, and *Plasmodium* (for review, see [90]), did not have an effect in mice and jirds experimentally infected with *E. granulosus* [91]. A small panel of di-*N*-aryl-diguanidino compounds was screened for efficacy against *E. multilocularis* metacestodes *in vitro*. Only those with a thiophene core group were active against metacestodes, while furans were not [92,93]. The most active compound,

DB1127 (**10**, Figure 10.2), was further assessed in mice. DB1127 was effective against AE when administered intraperitoneally but not when applied orally [92]. Thus, thiophene-diguanidino derivatives with improved oral bioavailability should be further developed.

A major focus in the search for anti-echinococcal activities has been antimalarial compounds. Artemisinin and artemisinin peroxides (ozonids), and mefloquine (**11**, Figure 10.2) and its enantiomers, were initially evaluated using *in vitro* cultured metacestodes [94,95]. *In vitro* treatment of *E. multilocularis* metacestodes with the antimalarials dihydroartemisinin and artesunate exhibited promising results. However, six weeks of *in vivo* treatment of mice infected with *E. multilocularis* metacestodes with these compounds had no effect. Combination treatments of both drugs with ABZ led to a measurable, but statistically nonsignificant, reduction in parasite weight compared to results with ABZ alone [95]. Further *in vitro* assessments of artemisinin derivatives using a series of amino-ozonids were carried out [94]. Three ozonids, namely OZ401, OZ455, and OZ491 containing an aminopropylether substructure, were the most potent, with IC_{50} values ranging from 11 to 14 µM. Cytotoxicity in different mammalian cell lines was observed only at higher concentrations. Transmission electron microscopy demonstrated complete destruction of the germinal layer after five days of drug exposure. Amino-ozonids have not been further pursued, since the concentration required for anti-echinococcal activity is not achieved in animals or humans. However, this class of compounds could be a valuable addition to the currently very limited arsenal of anti-echinococcal drugs, provided they are modified to increase bioavailability and pharmacokinetics to obtain increased exposure in the infected host.

Mefloquine, also widely used as an antimalarial, exhibits highly interesting *in vitro* and *in vivo* activities against *E. multilocularis* metacestodes. Previously, *in vitro* and *in vivo* studies demonstrated promising activities of mefloquine and mefloquine enantiomers in mice infected with young or adult stages of *Schistosoma mansoni* or *Schistosoma japonicum* [96,97]. Mefloquine was also shown to be active against *Opisthorchis viverrini in vitro* and in infected hamsters [98] and against larval and adult stages of *Brugia patei* and *Brugia malayi in vitro* [99]. *In vitro* treatment of *E. multilocularis* metacestodes resulted in detachment of large parts of the germinal layer from the inner surface of the laminated layer within a few hours [100]. Intraperitoneal application of mefloquine in secondarily infected mice (25 mg/kg, twice a week) resulted in a reduction in parasite weight that was similar to what was obtained with orally applied ABZ (200 mg/kg/d) [100]. More recent studies have shown that the success of oral application of mefloquine in mice is dose-dependent, and at a higher dosage (100 mg/kg/twice per week for 12 weeks), results in a reduction in parasite weight comparable to what is achieved by ABZ treatment (200 mg/kg /d) [101]. In the same study, two *Echinococcus* mefloquine-binding proteins were identified by affinity chromatography using mefloquine coupled to epoxy-activated Sepharose®, followed by SDS-PAGE and in-gel digestion LC–MS/MS. This resulted in the identification of *E. multilocularis* ferritin and cystatin as MEF-binding proteins. In contrast, affinity chromatography of human fibroblast extracts on mefloquine-sepharose matrices resulted in the identification of nicotinamide phosphoribosyl transferase. This indicates that mefloquine could potentially interact with different proteins in parasites and human cells [101].

Other antimalarial drugs with various degrees of efficacy against *E. multilocularis* metacestodes were identified more recently by repurposing the Medicines for Malaria Venture (MMV) Malaria Box [65].

This open-source library contains 400 commercially available chemicals that show *in vitro* activity against *Plasmodium falciparum*, and was provided by MMV free of charge. Primary PGI-assay-based screening was carried out at 10 µM, yielding 24 potentially interesting compounds that cause physical damage to metacestodes. Seven compounds retained their activity at 1 µM, but dose–response experiments showed that only two compounds exhibited an IC_{50} < 5 µM. After cytotoxicity assessment, only MMV665807 (**12**, Figure 10.2) was further assessed, and was shown to exhibit parasiticidal activity against germinal layer cell cultures. Transmission electron microscopy showed that MMV665807 primarily affected the structural organization of the mitochondrial matrix in the germinal layer tissue, and also caused an increased release of microvesicles into the laminated layer at a later stage. MMV665807 is a salicylanilide derivative, similar to the already commercially available niclosamide, which is used against adult-stage cestodes [102]. Niclosamide was shown to be also efficacious against various cancer cells *in vivo* and *in vitro* [103]. Unfortunately, when assessed in experimentally infected mice, both oral (gavage) and intraperitoneal application of MMV665807 did not result in reduced parasite burden [65]. However, currently, different formulations of MMV665807, designed to achieve increased plasma levels, are being assessed *in vitro* and *in vivo*.

Mathis et al. [104] were the first and only ones to describe a target-based *in silico* approach for the identification of novel compounds for echinococcosis treatment. They reported on the anti-echinococcal properties of clarithromycin (**13**, Figure 10.2), a macrolide antibiotic. Clarithromycin inhibits protein synthesis in bacteria by binding to the nascent peptide exit tunnel on the ribosome near the peptidyltransferase center of the large subunit rRNA [105]. Higher eukaryotes carry a guanine at position 2058 of both cytoplasmic and mitochondrial rRNAs, and the modification at this position had 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 predicts susceptibility as in bacteria [106], while the nucleus-encoded rRNA is characterized by a guanine at 2058. *In vitro* culture of *E. multilocularis* metacestodes in the presence of clarithromycin resulted in severely impaired growth and overall morphology of the germinal layer of these parasites. However, these results have not been followed up *in vivo*.

6.1.2 Anticancer Drugs

Doxorubicin, or hydroxydaunorubicin, a DNA-interacting drug used widely in the treatment of cancers, was one of the first anticancer drugs to be studied for its potential use against AE. Doxorubicin bound to polyisohexylcyanoacrylate nanoparticles (a colloidal biodegradable drug carrier) was applied in *E. mutlilocularis*-infected mice, which yielded a reduction of the parasite development in the liver and a reduced viability of the metacestode. In contrast, free doxorubicin or unbound nanoparticles had no antiparasitic activity [107]. However, due to the massive side effects that are generally encountered by doxorubicin, this treatment approach was not further pursued.

Isatin (1*H*-indole-2,3-dione) and its derivatives are responsible for a broad spectrum of biological activities. Among these, the cytotoxic and antineoplastic properties have been the most widely reported. The synthetic versatility of isatin has led to the generation of a large number of structurally diverse derivatives, due to its privileged scaffold. Several propargylic alcohols derived from isatin were

synthesized and the drug-induced morphological alterations in *E. multilocularis* -infected *Meriones unguiculatus* were described, documenting interesting antiparasitic properties [108].

Isoflavonoids, another class of antitumor agents with proven antiparasitic activities, are formed by plant tissue in response to physiological stimuli such as infectious agents. The isoflavonoid genistein, a major component of soya, is active against breast, prostate, skin, and colon cancer cell lines. Genistein also stimulates the synthesis of TGF-β, which itself inhibits cancer cell proliferation [109]. Besides other targets, genistein acts on several signaling pathways, inhibiting the activity of several kinases (tyrosine kinase, MAP kinase, ribosomal S6 kinase). In addition, genistein acts as a ligand for the estrogen receptor-beta; and upon long-term treatment, this could exert unfavorable effects [110]. Naguleswaran et al. [111] showed that besides genistein, several genistein derivatives that do not bind to the estrogen receptor-beta were also effective against *E. multilocularis* metacestodes *in vitro*, as well as against *E. granulosus* metacestodes and protoscoleces. These compounds could interfere in signaling, for instance, by interfering in the tyrosine kinase activity associated with the epidermal growth factor receptor identified in *E. multilocularis* [112], but the molecular mechanisms have not been elucidated. The anti-echinococcal efficacy of isoflavonoids has not been assessed *in vivo* to date. In contrast, tamoxifen (**14**, Figure 10.2), an antagonist of the estrogen receptor-alpha and used for the treatment of primary breast cancer, was shown to be moderately active against *E. multilocularis in vitro* [113], and to severely impair the growth of *E. granulosus* cysts in mice [114].

Another compound with antitumor effects, 2-methoxyestradiol (2-ME), an endogenous metabolite of estrogen, also induces severe and dose-dependent damage to *E. multilocularis* metacestodes *in vitro* [115]. Treatment of experimentally infected mice with 2-ME alone did not result in a reduction in parasite weight compared to the non-treated controls. The results achieved with the treatment of a combination of 2-ME and ABZ led to a substantial, but not statistically significant, increased reduction in parasite weight compared to ABZ treatment alone.

Protein kinases, especially serine/threonine and tyrosine kinases, activate a multitude of proteins and mediate signal transduction, cell growth, and differentiation. Kinases are known to play a crucial role in tumor progression, and they also are involved in the regulation of a plethora of cellular events in other diseases. From the list of the 20 most promising drug targets identified in the *E. multilocularis* genome [55], four are protein kinases. *E. multilocularis* metacestodes have been shown to express a wide range of signaling receptors including nuclear hormone receptor, TGF receptor, insulin receptor, epidermal growth factor receptor, and fetal growth factor receptor, which have been shown to be activated by either parasite- or host-derived ligands (reviewed in [116–118]). Pyridinyl imidazole compounds such as ML3403 and SB202190 are selective inhibitors of p38 mitogen-activated protein kinase (MAPK) *in vitro*, block pro-inflammatory cytokine production *in vivo*, and are implicated in the treatment of melanoma [119]. These pyridinyl imidazoles were identified to act as ATP-competitive inhibitors of MAPK of *E. multilocularis in vitro*, which resulted in the death of parasite vesicles at concentrations that did not affect cultured mammalian cells [120]. Other kinase inhibitors that were assessed in *E. multilocularis* vesicles were the Raf-inhibitor sorafenib and the MEK1/2 inhibitor PD184352 [121], which inhibited vesicle growth, but failed to exert parasticidal activity. The ABL-like kinase inhibitor imatinib (**15**, Figure 10.2), one of the first kinase inhibitors that was FDA approved as an anticancer drug, exhibited dose-dependent efficacy against *E. multilocularis* metacestodes,

protoscoleces, and stem cell cultures *in vitro* [122]. *E. multilocularis* metacestodes also express a kinase with significant homology to the Plk1 subfamily of Polo-like kinases in higher eukaryotes. Addition of BI 2536, a Plk1 inhibitor that has been tested in clinical trials against cancer, at concentrations as low as 20 nM significantly blocked the formation of metacestode vesicles from *Echinococcus* germinal cell cultures [123]. In addition, low concentrations of BI 2536 eliminated the stem cell population from mature metacestode vesicles *in vitro*, yielding parasite tissue that was no longer capable of proliferation. Thus, a series of kinase inhibitors that are candidate drugs (or are in use) for cancer treatment exhibit profound inhibitory properties on *E. multilocularis*. However, to the best of our knowledge, none of these findings has been reproduced in an animal model to date.

Cytostatic drugs can also exhibit effects that lead to increased proliferation and growth of *E. multilocularis* metacestodes *in vivo* [77]. It was shown that *in vitro* exposure of metacestodes to methotrexate and subsequent infection of mice with treated parasites led to massive growth and enhanced parasite proliferation, while navelbine and vincristine treatment had a slight negative impact on parasite proliferation.

Nitazoxanide, previously introduced as an anti-infective drug (see Section 10.6.1.1), also inhibits the proliferation of colon cancer cells *in vitro*, and Müller et al. [69] have shown that this happens by interfering with, and inhibiting, the activity of glutathione-*S*-transferase (GST) class π, an isoform overexpressed in many proliferating cells. In *E. granulosus* and *E. multilocularis*, the only GSTs characterized so far, have some sequence homologies to the mammalian class μ [124,125]. The catalytical properties of recombinant GST of *E. multilocularis* had, however, exhibited higher similarities to mammalian classes α and π, with, especially, a high conjugating activity on ethacrynic acid, another anticancer drug. In principle, GSTs may have two opposite effects on drugs, namely, by inactivating drugs or by activating ineffective prodrugs. Especially, the latter effects have been employed as an anticancer drug strategy and may be further developed against AE. In addition, these studies suggest that *Echinococcus* GST should be further investigated, and validated, as a potential drug target.

Another class of anticancer compounds that has attracted increasing attention in the past years are metallo-organic ruthenium complexes. Metallo-drugs were also shown to exhibit interesting antimicrobial properties, including activities against bacteria, trypanosomatids, *Toxoplasma*, and *Plasmodium* [126–129]. Two series of *η*6-areneruthenium(II) phosphite complexes were evaluated *in vitro* for their toxic potential against *E. multilocularis* metacestodes [130]. This screening identified several hydrolytically stable ruthenium complexes with *in vitro* toxicity for metacestodes in the range of nitazoxanide, also high cytotoxicity against rat hepatoma cells, but little toxicity for Vero cells and human fibroblasts. This indicates a certain potential for ruthenium compounds, but corresponding *in vivo* studies are still pending.

The first screening of a commercially available drug library was performed by Stadelmann et al. [113] by evaluating the efficacy of 426 compounds contained in an FDA-approved drug library. This library is composed of drugs against a diverse range of diseases, many of which are against viral infections and/or cancer. Initial screening at 20 μM revealed that seven drugs induced considerable metacestode damage, and further dose–response studies revealed that bortezomib (**16**, Figure 10.2), a proteasome inhibitor developed for the chemotherapy of myeloma, displayed high anti-metacestodal activity with

an EC_{50} of 0.6 µM, leading to an accumulation of ubiquinated proteins and unequivocally parasite death. In-gel zymography assays using *E. multilocularis* extracts demonstrated bortezomib-mediated inhibition of protease activity in a band of approximately 23 kDa, the same size at which the proteasome subunit beta 5 of *E. multilocularis* could be detected by Western blot. Treatment of BalB/c mice experimentally infected with *E. multilocularis* metacestodes with bortezomib led to reduced parasite weight, but to a degree that was not statistically significant, and it induced adverse effects such as diarrhea and neurological symptoms. Thus, this study identified the proteasome as a drug target in *E. multilocularis* metacestodes that can be efficiently inhibited and further investigations employing treatment adjustment and/or other proteasome inhibitors are necessary.

6.2 Drug Repurposing for the Discovery of Novel Compounds to Treat CE

Early studies carried out in experimentally infected mice and jirds assessed the *in vivo* effects of iodinated oil of thymol, ethyl-*N*-dimethyl ether of thymol fumarate, chloroguanide, rifampin, pentamidine isethionate, amphotericin B, suramin, and methotrexate on secondary CE. None of these compounds exhibited any meaningful reduction of cyst weight under the conditions used [91]. Other compounds were assessed for activity against *E. granulosus* protoscoleces such as cetrimide [131] and the ionophore monensin [132], but they were not effective against metacestodes. In contrast, levamisole and ivermectin, which are classically used against nematode infections, exhibited *in vitro* activities against protoscoleces as did benzimidazoles [133]. A novel prophylactic therapy approach that would avoid the formation of metastases due to spilling of *E. granulosus* protoscoleces was developed by exposing protoscoleces to praziquantel [30,31], or a combination of praziquantel and ABZ [30] prior to injection into mice. Since then, combined ABZ/praziquantel therapy in the treatment of human CE has been controversially discussed [32,134–136].

Benzimidazoles show variable efficacy. Albendazole and its metabolite ABZ-sulfoxide have been reported to be active against *E. granulosus* protoscoleces *in vitro* [137–139], but they act slowly over a period of several days to weeks. In contrast, MBZ and oxfendazole (fenbendazole-sulfoxide) seem to act more rapidly [138,140]. A combination of fenbendazole and netobimin, which is a prodrug of ABZ used as a veterinary anthelmintic, was shown to act synergistically against *E. granulosus* infection in rodents [141]. Experiments carried out in *E. granulosus*-infected sheep and goats suggested that oxfendazole may be as efficacious as ABZ, but does not require daily uptake of the drug because of its prolonged bioavailability [142]. Other benzimidazoles exhibiting interesting protoscolicidal and metacestodicidal activity are flubendazole [143] and nocodazole [144]. Flubendazole combined with nitazoxanide did also exhibit profound efficacy in *E. granulosus* protoscoleces and metacestodes *in vitro,* and in mice experimentally infected by intraperitoneal injection of protoscoleces [145]. *In vitro* treatments with another broad-spectrum anti-infective compound, thiazolide nitazoxanide [85], resulted in severe damage to protoscoleces and the germinal layer of the respective metacestodes within a few days [52], but nitazoxanide treatment in experimentally *E. granulosus*-infected sheep was not effective [146]. However, oxfendazole treatment, and a combination of oxfendazole and nitazoxanide, significantly decreased the number of fertile cysts and increased the number of degenerated cysts in sheep [146]. In addition, nitazoxanide did also not affect hydatid cyst development in mice [145]. Surprisingly, two case reports suggested that nitazoxanide may be an

effective treatment option in CE, particularly in patients with progressive disease who receive conventional therapy, but surely further studies need to be carried out to verify these findings in other patients [51,53].

The immunosuppressant drug cyclosporin A, which is employed mainly during the management of organ transplants, has reported activity against CE in the murine model. Cyclosporin has a profound effect when administered early (from two days) after infection, in five consecutive doses daily, resulting in reduced cyst numbers and mass after 20 weeks. When cyclosporin was administered 18 weeks postinfection, the wet weight was decreased by 42% compared to untreated controls. Transmission electron microscopy of the germinal membrane and laminated layer of late-treated *E. granulosus* revealed abnormalities in all cysts studied, whereas control and early-treated hydatid cysts were normal, indicating that cyclosporin exerts parasitostatic rather than parasiticidal effects [147].

Tamoxifen is a nonsteroidal selective estrogen receptor modulator binding to estrogen receptor-alpha, which is widely used against compounds for the treatment of primary breast cancer in premenopausal women and gynecomastia in men receiving hormonal therapy for prostatic carcinoma. At 10–50 µM, this compound impacted on *E. granulosus* protoscoleces and metacestode survival *in vitro*, and at a dose rate of 20 mg/kg of body weight, tamoxifen induced protection against the infection in mice. In the clinical efficacy studies, a reduction in cyst weight was observed after the administration of 20 mg/kg in mice with cysts developed during three or six months, compared to that of those collected from control mice [114]. Tamoxifen was also shown to be active against the cestodes *Taenia crassiceps* and *T. solium* [148,149]. The activity of another anticancer drug, the proteasome inhibitor bortezomib, was demonstrated recently. Bortezomib was shown to exhibit considerable *in vitro* activity by eliciting endoplasmic reticulum stress and autophagy in *E. granulosus* protoscoleces and metacestodes, but no *in vivo* studies have been reported so far [150]. In addition, bortezomib was evaluated in combination with the kinase inhibitor rapamycin, and a synergistic effect on protoscolece viability could be observed with the combination. Rapamycin is a polyketide macrolide that binds to FK506-binding proteins, which are involved in protein folding, and known to be targets for antiproliferative drugs. The rapalogs rapamycin, FK506 (tacrolimus), and everolimus were reported to exhibit protoscolicdal effects *in vitro*, and synergistic scolicidal actions were observed during combined therapy with rapalogs plus cyclosporine [150]. *In vivo* studies on these rapalogs have not been reported to date.

Recent studies investigated the protoscolicidal effects of chenodeoxycholic acid (CDCA) and sodium arsenite *in vitro* using eosin staining and caspase detection assays to monitor apoptosis. CDCA is a bile acid that has been therapeutically tested against hepatitis C (in combination with bezafribate), and is used against constipation and against cerebral cholesterosis. Dose-dependent mortality and induction of apoptosis in protoscoleces was noted at concentrations of 500–3000 µM after a few days of treatment [151]. Sodium arsenite has strong teratogenic and carcinogenic effects, and is not surprisingly toxic for protoscoleces at lower concentrations (16 µM) [152]. However, the reported toxicity and the strong adverse reactions expected through treatment with sodium arsenite render its application rather unlikely.

The activities of two antidiabetic drugs against secondary CE in mice were demonstrated. Glibenclamide is a second-generation sulfonylurea receptor inhibitor, which has been shown to be active against protozoan parasites [153]. Metformin is a plant-derived antihyperglycemic and potential

anticancer agent which may exert its antiproliferative effects via the induction of energetic stress and activation of AMP-activated protein kinase [154]. No *in vivo* data is available for glibenclamide to date, but oral administration of metformin (50 mg/kg/d) in *E. granulosus*-infected mice was highly effective in reducing the weight and number of parasite cysts, yet its combination with the lowest recommended dose of ABZ (5 mg/kg/d) was even more effective [153].

Natural products obtained from essential oils from a variety of medicinal plants have been frequently studied as protoscolicidal agents, either to replace 20% NaCl and 95% ethanol during PAIR, to reduce metastasis formation by protoscoleces spillage during surgery, or to reduce cyst load by *E. granulosus* metacestodes. Of all the natural herb products tested, thymol and carvacrol (**17**, Figure 10.2), two isomers which differ only by the positioning of a hydroxyl group appear to represent the most promising option. Thymol and carvacrol are the main components of essential oils of *Thymus vulgaris* and *Origanum vulgare*, and they have reported antibacterial and antifungal properties. Prior to the development of other anthelminthics, thymol was used to treat hookworm infections in the United States [155]. Elissondo et al. demonstrated the efficacy of thymol against *E. granulosus* protoscoleces [156]. Due to its apparent lack of toxicity combined with good efficacy, it was proposed to be used as a scolicidal agent during hydatid cysts surgery and/or PAIR [157]. Further investigations on thymol-based therapy against murine CE demonstrated promising chemoprophylactic and therapeutic efficacy that was comparable to ABZ treatment, and the authors suggested that thymol could be applicable as an alternative treatment in human CE [158]. Similarly, Fabbri et al. demonstrated profound *in vitro* and *in vivo* efficacy of carvacrol against *E. granulosus* metacestodes in mice, comparable with ABZ, suggesting that this compound could be applied as a potential alternative treatment option [159].

Mentha piperita and *Mentha pulegium* essential oils were studied for their efficacy against protoscoleces and metacestodes *in vitro* [160]. *M. pulegium* essential oil induced dose- and time-dependent protoscolicidal effects, as well as loss of turgor in metacestodes maintained *in vitro*. These findings were substantiated by scanning and transmission electron microscopy studies of protoscoleces and germinal layer. The main compound in *M. pulegium* essential oil is piperitone oxide, and it was suggested, but unfortunately not conclusively demonstrated, that piperitone oxide could be responsible for protoscolicidal and metacestodicidal effects. Other natural products that were described to have significant anti-protoscolex activity are derived from endophytic *Pestalotiopsis* sp. [161], from the circassian walnut *Juglans regia* [162], *Myrtus communis L*. essential oil [163], and *Nectaroscordum tripedale L*. leaf extract [164]; but in all these studies, the corresponding active substances have not been determined.

7 Where to Go from Here?

For many of the compounds presented here, no *in vivo* studies were published after promising *in vitro* activities had been observed. This could be a result of (i) lack of project financing, (ii) lack of specificity and toxicity of the compound, or (iii) the authors wanted to refrain from publishing “negative” results, which is, unfortunately, a commonly observed fact. Several substances were not pursued further, even though they looked promising in *in vivo* mouse trials, mainly due to side effects and toxicity. Others were not followed up most likely because of financial constraints.

A few drugs were, however, applied in human patients. Amphotericin B was used as a salvage treatment in humans, but is not recommended due to its intravenous route of administration and resulting nephrotoxicity [49]. Nitazoxanide was also applied against human AE, but it did not prove to be effective [49,50]. Selected compounds received further attention regarding their formulation and mode of application in the *in vivo* mouse model: DB1127 was active when injected intraperitoneally, but not *per os* [92]. DB1127 was not further followed up because its prime indication project on protozoan parasites was discontinued, and data on bioavailability and pharmacokinetics were lacking [33]. Mefloquine was active in the mouse model when applied orally at a dosage of 100 mg/kg [101], but did not prove to be acting in a parasiticidal manner. Till date, there is reluctance to the long-term treatment of patients with AE using mefloquine, due to expected neurological side effects [165]. Thymol, an essential oil extracted from the plants *T. vulgaris* and *O. vulgare*, was successfully applied as an oral formulation in mice and exhibited a synergistic effect together with albendazole in both AE and CE mouse models. However, for AE, these findings on thymol could not be reproduced (own unpublished results). It is not known whether thymol acts parasiticidal and no studies have yet been performed on the potential toxicity of long-term application of thymol or its effects in the human patient [87].

Thus, none of the approaches carried out to date have identified alternatives with improved properties compared to the benzimidazoles used to date. However, some promising compounds (MMV665807, mefloquine, and thymol) are still being further investigated. Novel treatment options that act parasiticidal are still lacking. Thus, further screening efforts should focus on the screening of additional drug libraries to identify better compounds with increased efficacy, selective toxicity, and improved safety; and more biochemical and molecular studies are needed to identify relevant drug targets [33]. Combining drugs with different mechanisms of action, as has been done in a few instances, could be a possibility to improve treatment efficacy.

E. multilocularis and *E. granulosus* are highly adapted to a parasitic life style. Crucial genes and entire pathways for the *de novo* synthesis of pyrimidines, purines, and amino acids are absent in the genome, and genes for fatty acid and cholesterol *de novo* synthesis are largely missing [55]. Thus, these parasites need to acquire amino acids, lipids, purines, and other metabolites from their host, and transcripts coding for respective proteins involved in uptake and transport of these essential components are upregulated in the metacestode stage. These auxotrophies should be exploited for the development of novel therapeutic options. In addition, cestode parasites, similar to other helminths, express the malate dismutation pathway, with the anaerobic NADH-fumarate reductase system as a predominant component in the respiratory chain of *E. multilocularis*, and thus providing a unique opportunity to target the energy metabolism [45]. However, while much should/could be done, finances for building up a research program on novel drugs for echinococcosis are difficult to acquire, and funding on this topic is not being regarded as a priority, neither by private nor public authorities. Hopefully, this will change in the near future.

Acknowledgments

The authors acknowledge the support of the Swiss National Science Foundation (grant no. 160108), the Swiss State Secretariat of Education and Science (grant no. C15.0034), the Bangerter Rhyner Foundation (Switzerland), and the Fondation Sana (Switzerland).

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