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</tr>
</thead>
<tbody>
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<tr>
<td>Citation</td>
<td>Parasitology International, 75, art.no.102004; 2020</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2019-10-31</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10069/39549">http://hdl.handle.net/10069/39549</a></td>
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Mitochondrial complex III in larval stage of *Echinococcus multilocularis* as a potential chemotherapeutic target and *in vivo* efficacy of atovaquone against primary hydatid cysts

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**ARTICLE INFO**

**Keywords:**
- *Echinococcus multilocularis*
- Oxidative phosphorylation
- Fumarate respiration
- Mitochondrial complex III
- Atovaquone
- Drug target

**ABSTRACT**

*Echinococcus multilocularis* employs aerobic and anaerobic respiration pathways for its survival in the specialized environment of the host. Under anaerobic conditions, fumarate respiration has been identified as a promising target for drug development against *E. multilocularis* larvae, although the relevance of oxidative phosphorylation in its survival remains unclear. Here, we focused on the inhibition of mitochondrial cytochrome b\(_6\) complex (complex III) and evaluated aerobic respiratory activity using mitochondrial fractions from *E. multilocularis* protoscoleces. An enzymatic assay revealed that the mitochondrial fractions possessed NADH-cytochrome c reductase (mitochondrial complexes I and III) and succinate-cytochrome c reductase (mitochondrial complexes II and III) activities in the aerobic pathway. Enzymatic analysis showed that atovaquone, a commercially available anti-malarial drug, inhibited mitochondrial complex III at 1.5 nM (IC\(_{50}\)). In addition, culture experiments revealed the ability of atovaquone to kill protoscoleces under aerobic conditions, but not under anaerobic conditions, indicating that protoscoleces altered their respiration system to oxidative phosphorylation or fumarate respiration depending on the oxygen supply. Furthermore, combined administration of atovaquone with atpenin A5, a quinone binding site inhibitor of complex II, completely killed protoscoleces in the culture. Thus, inhibition of both complex II and complex III was essential for strong antiparasitic effect on *E. multilocularis*. Additionally, we demonstrated that oral administration of atovaquone significantly reduced primary alveolar hydatid cyst development in the mouse liver, compared with the untreated control, indicating that complex III is a promising target for development of anti-echinococcal drug.

**1. Introduction**

Alveolar echinococcosis (AE) is caused by the larval stage of *Echinococcus multilocularis* and is one of the most harmful and life-threatening helminth. This parasite is maintained between two different hosts. The definitive hosts are feral carnivores such as foxes, wolves, and dogs. The fully-grown parasites in the small intestine of definitive hosts release their eggs into the feces of the definitive host. Accidental ingestion of the eggs by intermediate hosts such as small rodents, leads to release of an infective larva (oncosphere) upon stimulation with gastric juice and bile in the intestinal lumen, and the oncosphere migrates to major organs via the circulatory system to form larval cysts.

The growth of larval cysts in the liver leads to life threatening conditions such as organ dysfunction in their intermediate hosts several years after infection. These larval cysts primarily consist of an outer acellular laminated layer and an inner germinal layer, which may give rise to brood capsules. Protoscoleces are produced from the inner wall of the brood capsules by asexual division [1,2].

The first choice for chemotherapy of AE is benzimidazole derivatives such as albendazole (ABZ), although these agents are parasitostatic rather than parasitocidal against *E. multilocularis* larvae [3]. Since the discovery of ABZ in the 1970s, no new drugs for AE have been identified. Moreover, radical surgery for advanced AE is typically difficult [1,4], and thus the development of effective anti-echinococcal...
Our group has focused on the mitochondrial respiratory chain, particularly the NADH-fumarate reductase system (fumarate respiration), of parasites as a potential drug target [7,8]. Studies of Ascaris suum revealed that fumarate respiration is composed of complex I (NADH-quinone reductase), complex II (quinol-fumarate reductase, QFR), and low-potential electron mediator, rhodoquinone (RQ). Low-potential RQ transfers a reducing equivalent of NADH via complex I to complex II, and succinate is ultimately produced from fumarate by the QFR activity of complex II (Fig. 1). The advantage of this system is that ATP can be synthesized using proton-pumping activity of complex I and ATP synthase (complex V) even in the absence of oxygen. QFR catalyzes the reduction of fumarate to succinate under anaerobic conditions, whereas succinate-quinone reductase (SQR), used in mammalian systems, oxidizes succinate in the opposite direction under aerobic conditions as a TCA cycle member. Parasitic helminths conduct fumarate respiration, which is highly adapted to anaerobic conditions [9,10]. Several studies have suggested the possible existence of an anaerobic respiratory chain in *E. multilocularis* [11,12]. *E. multilocularis* possesses NADH-fumarate reductase activity as the predominant activity as a specific adaptation to anaerobic environments, and this unique respiratory system is a promising target for chemotherapy of AE [8].

Complex I, which is one of the components of fumarate respiration, is a candidate of drug target. Rotenone, quinazoline and its derivatives are representative inhibitors of complex I, which exhibited anti-echinococcal activity under *in vitro* culture [8]. However, they exhibited strong toxicity in mammalian cells [13]. Thus, we have focused on complex II, which is the other component of this system. Potent mammalian complex II inhibitor, atpenin A5, was found to inhibit complex II of *E. multilocularis* in nanomolar range [7,14]. Interestingly, ascocuranone, which inhibits cyanide-insensitive alternative oxidase of African trypanosome, *Trypanosoma brucei* [15], also inhibits *E. multilocularis* complex II [7].

Previous studies have shown that both oxidative phosphorylation and NADH-fumarate reductase systems function as respiratory chains in the lung fluke, *Paragonimus westermani*, which, like *E. multilocularis*, inhabits cysts surrounded by the host tissues [16,17]. However, little is known about the importance of oxidative phosphorylation in *E. multilocularis* despite the presence of cyanide-sensitive NADH-oxidase, ubiquinol-oxidase, and tetramethyl-p-phenylenediamine (TMPD) oxidase [8]. Oxidative phosphorylation is generally composed of complexes I, II, III, IV, and V. Complex I accepts electrons from NADH and passes them to ubiquinone (UQ); Complex II, which functions as a succinate quinone reductase (SQR), receives electrons from succinate. UQ transfers electrons to complex III, which passes them to complex IV via cytochrome c. The electrons are used to reduce an oxygen molecule to oxygen ion, which attracts hydrogen ions to form water. At the same time, complexes I, III, and IV function as proton pumps to produce a proton gradient that drives ATP synthase.

**Fig. 1.** Schematic representation of the aerobic and anaerobic respiration system in *A. suum*. The NADH-fumarate reductase system (fumarate respiration) is composed of complex I (NADH-quinone reductase), low-potential rhodoquinone (RQ), and complex II (quinol-fumarate reductase, QFR). In this system, electrons from NADH are transferred to RQ via the NADH-RQ reductase activity of mitochondrial complex I, and then transferred to fumarate via the QFR activity of mitochondrial complex II through the quinone binding site. Electrons are transferred in complex I coupled with proton transport across the mitochondrial inner membrane to generate ATP. Oxidative phosphorylation (aerobic respiration) is generally composed of complexes I, II, III, and IV. Complex I accepts electrons from NADH and passes them to ubiquinone (UQ); Complex II, which functions as a succinate quinone reductase (SQR), receives electrons from succinate. UQ transfers electrons to complex III, which passes them to complex IV via cytochrome c. The electrons are used to reduce an oxygen molecule to oxygen ion, which attracts hydrogen ions to form water. At the same time, complexes I, III, and IV function as proton pumps to produce a proton gradient that drives ATP synthase.
respiratory inhibitors. Furthermore, to investigate whether complex III of *E. multilocularis* plays a vital role in survival of the parasite, thereby serving as a drug target, the effect of ATV and ABZ on larval cysts was examined in vivo.

2. Materials and methods

2.1. Isolation of *E. multilocularis* protoscoleces

We used the Nemuro strain of *E. multilocularis*, which is maintained at the Hokkaido Institute of Public Health (Sapporo, Japan). Mature larval parasites showing protoscoleces formation were obtained from cotton rats (*Sigmodon hispidus*) more than four months after oral infection with 50 eggs [27]. To isolate protoscoleces, the cysts of *E. multilocularis* were minced with scissors, passed through a metal mesh, and washed repeatedly with physiological saline until the host materials were thoroughly removed [8].

2.1.1. Preparation of mitochondrial fraction from *E. multilocularis* and washed repeatedly with physiological saline until the host mate-

2.1.2. Preparation of mitochondrial fraction from porcine heart

The enriched mitochondrial fraction of *E. multilocularis* protoscoleces was prepared essentially as described previously [8]. Briefly, the parasite materials were homogenized with a motor-driven homogenizer (six passes three to four times). The homogenate was diluted with the mitochondrial preparation buffer (210 mM mannitol, 10 mM sucrose, 1 mM disodium EDTA, and 50 mM Tris-HCl [pH 7.5]) supplemented with 10 mM sodium malonate to 5 times the volume of the original protoscoleces sediment and then centrifuged at 800 × g for 10 min (4 °C) to precipitate cell debris and nuclei. The supernatant was then centrifuged at 8000 × g for 10 min (4 °C) to obtain the mitochondrial pellet. The pellet was re-suspended in mitochondrial preparation buffer (without malonate) and centrifuged at 8000 × g for 10 min (4 °C). The enriched mitochondrial fraction was suspended in mitochondrial preparation buffer without malonate.

2.1.3. Preparation of mitochondrial fraction from porcine heart

All subsequent procedures were carried out at 4 °C. Porcine heart muscles were sectioned into several pieces, which were then added into 20 mM sodium phosphate buffer (pH 7.4) in a weight-to-volume ratio of 100 g/300 mL and homogenized in a high-speed blender for 90 s. The homogenate was immediately centrifuged for 20 min at 3000 × g and the supernatant containing the mitochondria was percolated through 8-layer gauzes. The supernatants were centrifuged at 18,000 × g for 60 min. The supernatant was discarded and the precipitate was suspended in 50 mM Tris-HCl (pH 8.0). The suspension was centrifuged using an ultracentrifuge at 120,000 × g for 40 min. The supernatant liquid was discarded, and the precipitate containing the mitochondrial fraction was suspended in 0.1 M borate-phosphate buffer (pH 7.2) [28,29].

2.2. Enzyme assays

All enzyme assays using mitochondrial fractions were performed in 0.5 or 1 mL reaction mixtures at 25 °C. During the assay using the mitochondrial suspension, it was thawed at room temperature, and then returned into a deep freezer to refreeze it. The sample refroze and was thawed again after one hour. This process was performed as a freezing and thawing before the assay in order to be permeable the mitochondrial membrane to the solutes. The reagents used in each assay were mixed with the reaction buffer (30 mM potassium phosphate, 1 mM MgCl2, pH 7.5). The final mitochondrial protein concentration was 50 μg/mL of reaction mixture. NADH-deQ (decyldihydroquinone) reductase, SQR, QFR, and NADH oxidase activities were measured essentially as described previously [8]. Succinate-cytochrome c reductase activity (complexes II and III) was determined by monitoring the absorbance change of reduced cytochrome c at 550 nm (ε = 19 × 10^3 M^−1 cm^−1) (SHIMADZU spectrophotometer UV-3000, Kyoto, Japan) in the presence of 50 μM cytochrome c and 2 μM potassium cyanide (KCN). The reaction was initiated by the addition of disodium succinate to a final concentration of 10 mM to the mixture. NADH-cytochrome c reductase activity (complexes I and III) was determined by the same method as used for succinate-cytochrome c reductase activity assay in the presence of 100 mM Na-malonate, 50 μM cytochrome c, and 2 mM KCN. The reaction was started by the addition of NADH to a final concentration of 50 μM to the mixture.

We determined the 50% inhibitory concentration (IC_{50}) values of representative complexes I, II, and III inhibitors against the specific activities of mitochondrial respiratory enzymes in *E. multilocularis* protoscoleces. The inhibitory activities for compounds were efficiently screened as follows: First, the inhibitory activities of compounds were checked at 10 μM. Second, to determine the inhibitory activities of compounds with a rate of inhibition > 90%, a ten-fold dilution series was used. Finally, we chose compounds with a high inhibition rate, and the IC_{50} of each compound was determined by calculating approximation lines which were made from 3 or more points of anteroposterior concentration of 50% inhibition. Antimycin A (Sigma, St. Louis, MO, USA), myxothiazol (Sigma), azoxystrobin (Sigma), and atovaquone (Tokyo Chemical Industry, Tokyo, Japan) were tested as representative complex III inhibitors in the assays. Rotenone (Wako Pure Chemical Industries, Osaka, Japan), a complex I inhibitor, and atenpin A5 (Kitsato University), which is a complex II inhibitor, were used in this study. IC_{50} value of atenpin A5 for complex III was determined on the basis of NADH-cytochrome c reductase activity (complexes I and III), since succinate-cytochrome c reductase activity (complexes II and III) was inhibited by the potent inhibitory effect of atenpin A5 on complex II.

2.3. In vitro treatment of living *E. multilocularis* protoscoleces

Protoscoleces were cultured in CMRL 1066 medium (Gibco, Grand Island, NY, USA) containing 23 mM HEPES, 0.5% (w/v) D (+)-glucose, 0.4 mM sodium taurocholate (Wako Pure Chemical Industries), 0.5% (w/v) yeast extract (Difco Laboratories, Detroit, MI, USA), 57 mM sodium hydrogen carbonate, 2 mM glutamine (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Pen Strep). Half of the medium was replaced on day 3. In the anaerobic experiment of the parasite cultures, a 6-well plate was sealed in a plastic container with oxygen detection agent and oxygen scavenger (Aneromeito®, Nissui Pharmaceutical, Tokyo, Japan) to maintain the oxygen concentration under 0.3% at 37 °C. This culture condition was also applied during *in vitro* treatment of the parasite by the inhibitors. To examine the efficacy of chemical compounds against living *E. multilocularis* protoscoleces, the parasites were treated with rotenone, atenpin A5, or ATV at a final concentration of 50 μM in the culture medium. The control group was supplemented with 0.5% (v/v) dimethyl sulfoxide, and all conditions were assayed in triplicate. Two hundred microliters of each culture medium with suspended protoscoleces were taken and transferred into a 24-well plate to determine the viability of protoscoleces using the trypan blue exclusion assay [8,30]. > 170 protoscoleces per well were stained with trypan blue and were microscopically counted (40 × magnification).

2.4. In vivo studies on the efficacy of ATV in experimentally infected BALB/c mice

Experiment A: The *in vivo* effects of ATV were evaluated and compared with those of the control and standard oral ABZ treatments to investigate the importance of complex III as a drug target. BALB/c mice (female, 9 weeks old, average body weight 25 g, and daily average food intake of 4.0 g) were infected orally with 100 μL of a 200 eggs-containing suspension prepared from the feces of an *E. multilocularis* infected dog. Three mice were necropsied to confirm the presence of cysts.
(1–2 mm diameter, 1–2 mm long) in the liver at 4 weeks post-infection. After confirming cyst formation, mice were randomly allocated into 3 groups of 6 animals each and treated with ATV or ABZ; untreated mice were used as controls. ATV and ABZ were mixed into the feed at a rate of 133 mg/100 g and 125 mg/100 g, respectively. Each compound was blended with pulverized feed by a waring blender until it reached a consistency [31]. Water was added to the clayish feed, and pellets (2 × 2 × 3 cm) were shaped with a spatula. The pellets were dried in an incubator (50 °C, 6–8 h). In a preliminary experiment, we verified that IC50 of ATV for succinate-cytochrome c reductase in E. multilocularis was not affected by heating at 50 °C, 8 h. The treatments were performed for 12 weeks by feeding the mice with the drug-mixed feed ad libitum. Necropsy was performed at the end of drug administration and the proportion of cysts on the liver surface was measured with digital image analysis software (ImageJ, Bethesda, MD, USA) to evaluate cyst growth.

Experiment B: We also assessed the effectiveness of prophylactic administration of ATV in mice. Fifty-eight BALB/c mice (female, 7 weeks old) were divided into 3 groups as follows: 20 mice in the control group, 22 mice in the ATV group, and 16 mice in the ABZ group. ATV and ABZ were mixed into the feed as described above. Four days prior to infection, mice were orally administered ATV or ABZ by feeding of the drug-mixed feed ad libitum. For half of the mice in each group, administration of ATV and ABZ was terminated on the day of infection; these mice were allocated into the “experiment B-1 group”. For the remaining mice in each group, administration of ATV and ABZ was continued for 4 weeks (experiment B-2). The control group consumed normal feed. All animals had access to water ad libitum. Three hundred eggs prepared from the feces of an E. multilocularis-infected dog were administrated orally. All mice were sacrificed 4 weeks after infection and necropsies were performed. The number of AE cysts on the liver in each mouse was counted. The results were analyzed by the Kruskal–Wallis test. P < .05 was considered significant. Experiments were carried out according to Hokkaido Institute animal welfare regulations: approval number K26-3 and K29-4.

3. Results

3.1. Enzyme activities of E. multilocularis protoscoleces mitochondria

The specific enzyme activities in the mitochondrial aerobic and anaerobic respiratory chains of E. multilocularis protoscoleces are shown in Table 1. NADH-decylrhodoquinone reductase activity was 65.4 nmol/min/mg under aerobic conditions. SQR activity was 236 nmol/min/mg. QFR activity, which is the reverse reaction of the SQR activity of complex II under anaerobic conditions, was 103 ± 17.5 nmol/min/mg. NADH-fumarate reductase activities were not detected in porcine mitochondria, which served as a control of aerobic respiratory chain of mammals. The specific activity of NADH-cytochrome c reductase (complexes I and III) was 48.1 nmol/min/mg and that of succinate-cytochrome c reductase (complexes II and III) was 65.6 nmol/min/mg, respectively. NADH oxidase activity was 18.4 nmol/min/mg, and was suppressed by 100 mM malonate, which blocks the leakage of electrons from complex II, and oxidase inhibitor 2 mM KCN.

3.2. Effects of the respiratory inhibitors on mitochondria of E. multilocularis protoscoleces

Next, the effect of well-known specific inhibitors of mammalian respiratory chain on complex I, II and succinate-cytochrome c reductase (complexes II and III) was examined (Table 2). The complex I inhibitor rotenone showed IC50 0.74 μM on NADH-rodhoquinone reductase activity, while atpenin A5, which is a potent and specific inhibitor of complex II, inhibited SQR activity at IC50 values of 0.69 μM and 1.5 nM, respectively indicating that ATV inhibited E. multilocularis complex III at an extremely low concentration. The IC50 of ATV for succinate-cytochrome c reductase was considerably lower than that of a well-known complex III inhibitor, antimycin A. These results indicate clearly the presence of aerobic respiratory chain in addition to fumarate respiration in E. multilocularis protoscoleces mitochondria.

### Table 1

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sp acta (nmol/min/mg of protein) (mean SD)</th>
<th>E. multilocularis protoscoleces</th>
<th>Porcine</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH-decyl rhodoquinone reductase (complex I)</td>
<td>65.4 ± 9.7</td>
<td>111 ± 10.5</td>
<td></td>
</tr>
<tr>
<td>Succinate-quione reductase: SQR (complex II)</td>
<td>236 ± 25.3</td>
<td>382 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>Quinol-fumarate reductase: QFR (complex II) under anaerobic</td>
<td>103 ± 17.5</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>NADH-fumarate reductase (complex I and II) under anaerobic</td>
<td>35.5 ± 4.0</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>NADH-cytochrome c reductase (complex I and III)</td>
<td>48.1 ± 6.1</td>
<td>947 ± 20.5</td>
<td></td>
</tr>
<tr>
<td>Succinate-cytochrome c reductase (complex II and III)</td>
<td>65.6 ± 9.6</td>
<td>283 ± 17.8</td>
<td></td>
</tr>
<tr>
<td>NADH oxidase (complex I, III and IV) with 2 mM KCN and 100 mM malonate</td>
<td>18.4 ± 1.3</td>
<td>568 ± 34.9</td>
<td></td>
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<tr>
<td>with 2 mM KCN</td>
<td>4.4 ± 1.4</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

N.D.: Not detected.

* Specific activities were obtained from at least three independently isolated mitochondria.

### Table 2

Inhibitory effect of representative quinone-binding site inhibitors of E. multilocularis protoscoleces mitochondria.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 (μM)</th>
<th>(Complex I)</th>
<th>(Complex II)</th>
<th>(Complex II and III)</th>
</tr>
</thead>
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<tr>
<td>Rotenone</td>
<td>0.74 ± 0.13</td>
<td>&gt; 30</td>
<td>&gt; 30</td>
<td>&gt; 30</td>
</tr>
<tr>
<td>Atpenin A5</td>
<td>&gt; 30</td>
<td>0.042 ± 0.008</td>
<td>&gt; 20</td>
<td>&gt; 30</td>
</tr>
<tr>
<td>Azoxystrobin</td>
<td>&gt; 30</td>
<td>&gt; 30</td>
<td>&gt; 30</td>
<td>&gt; 30</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>&gt; 30</td>
<td>&gt; 30</td>
<td>0.0068 ± 0.0001</td>
<td>&gt; 30</td>
</tr>
<tr>
<td>Atovaquone</td>
<td>26 ± 1.0</td>
<td>0.69 ± 0.02</td>
<td>0.0015 ± 0.0009</td>
<td>&gt; 30</td>
</tr>
</tbody>
</table>

* NADH-cytochrome c reductase (Complex I and III).
3.3. Effects of the respiratory inhibitors on the viability of E. multilocularis protoscoleces under aerobic and anaerobic conditions

Since the succinate-cytochrome c reductase and NADH-cytochrome c reductase activities in the aerobic pathway were confirmed, in vitro experiments with protoscoleces were performed under aerobic and anaerobic conditions with each inhibitor (Fig. 2). The viability of E. multilocularis protoscoleces was progressively reduced during in vitro treatment of the parasite with 50 μM rotenone, atpenin A5, ATV, and ATV plus atpenin A5. Rotenone killed E. multilocularis protoscoleces after similar days of treatment under both conditions. It should be noted that the effects of atpenin A5 and ATV were different between the aerobic and anaerobic conditions. Atpenin A5 showed an antiparasitic effect of only 30% elimination on day 7 under aerobic conditions, but exhibited potent parasite-killing activities against E. multilocularis protoscoleces under anaerobic culture conditions and completely eliminated the parasites on day 5. In contrast, no killing was observed by ATV under anaerobic condition, while ATV eliminated E. multilocularis protoscoleces completely by day 7 under aerobic condition. Interestingly, strong elimination ability was observed by co-administration of atpenin A5 with ATV under both conditions. These results indicate that inhibition of mitochondrial complex II under hypoxic conditions had high anti-echinococcal effects, and complex III was essential for the survival of E. multilocularis protoscoleces under aerobic conditions.

3.4. Effect of ATV treatment in mice orally infected with E. multilocularis eggs

Since ATV showed significant inhibition of parasite complex III, its effects on mice orally infected with E. multilocularis eggs as a natural infection model was examined. In experiment A, suppression of E. multilocularis cyst development on the liver by ATV was examined in mice following oral infection of the eggs (Fig. 3A). A significant reduction in the total surface area of the cysts was observed in the ATV-treated group compared with that in the untreated control group (p = .048). The standard oral ABZ resulted in a significant and stable reduction in the surface area of cysts compared with that in the control group (p = .009) and ATV groups (p = .028).

In experiment B, to evaluate the prophylactic effects of therapy, oral administration of ATV was started 4 days prior to infection (Fig. 3B, experiment B-2). The number of cysts was significantly reduced in mice administered ATV for up to 4 weeks after infection compared with that in the control group (p = .001). ATV and ABZ showed similar effects (Fig. 3B, experiment B-2). ATV showed no significant efficacy when ATV administration was terminated at the time of infection (experiment B-1) compared with the control (data not shown), although the number of cysts was significantly reduced in mice administered ABZ. These results suggest that ATV suppresses the growth of larvae (oncosphere) after attachment to the liver rather than staying in the blood vessels or intestinal mucosa.

4. Discussion

In this study, biochemical analysis and in vitro experiments showed that E. multilocularis protoscoleces possesses aerobic respiratory chain in addition to fumarate respiration. This mammalian type respiration includes complex III, which was inhibited by ATV in the nanomolar range (IC50: 1.5 nM). We also demonstrated that ATV significantly reduced the growth of E. multilocularis larval cysts in vivo using orally infected mice, which is a natural infection model. These results suggest that complex III of the respiratory system plays an important role in the survival of E. multilocularis in the intermediate host.

We previously suggested that the fumarate respiratory system of E. multilocularis is a promising target for the development of novel selective anti-echinococcal drugs [7,8]. Notably, in the present study, we clearly identified NADH oxidase activity (complexes I, III and IV) as well as NADH-cytochrome c (complexes I and III) and succinate-cytochrome c (complexes II and III) reductase activities in E. multilocularis protoscoleces. This result indicates that oxidative phosphorylation, which involves the mitochondrial complexes I, II, III, and IV, plays an important role in the survival of E. multilocularis protoscoleces in addition to anaerobic fumarate respiration. NADH-oxidase activity observed in this study was two times higher than that reported previously [8]. It might be possible that small differences in oxygen availability in parasite habitats between the present and previous preparations resulted in such different enzyme activities.

It has been reported that rhodoquinone (RQ) is utilized in E. multilocularis as a major quinone component [8]. However, E. multilocularis may deftly use RQ and ubiquinone (UQ) to transport electrons, since the genes for oxidative phosphorylation as well as ubiquinone biosynthesis [32] have been identified in the parasite genome. It should be noted that the mitochondria of adult P. westermani that possess anaerobic and aerobic respiratory systems contain both RQ-10 and UQ-10 [16,17]. Further studies are needed to evaluate these possibilities in E. multilocularis.
multilocularis.

We found a difference in the sensitivity to inhibitors when E. multilocularis protoscoleces were cultured in aerobic and anaerobic culture conditions. Under aerobic conditions, ATV displayed strong anti-parasitic ability against protoscoleces, while atepin A5, a specific complex II inhibitor, had an antiparasitic effect with only 30% elimination on day 7. In contrast, when treated under anaerobic conditions, ATV did not eliminate E. multilocularis protoscoleces, while atepin A5 completely eliminated the larvae on day 5. These results indicate the presence of dual respiratory chains in E. multilocularis protoscoleces mitochondria, classical mammalian type and fumurate respiration, which the parasite uses depending on the oxygen availability. Thus, inhibition of the complex II alone is not enough for killing E. multilocularis, and additional inhibition of complex III is required for effective treatment.

Dynamic changes in the respiratory system have been identified in both helminths and protozoans during their life cycles to adapt to changes in environmental oxygen tension. Oxidative phosphorylation is used in the free-living infective larva of A. suum under high oxygen conditions, and is completely replaced by fumurate respiration in the adult stage A. suum living in the small intestine, which is an anaerobic environment [14,33]. Aerobic mitochondrial metabolism in the free-living and juvenile parasitic stages of Fasciola hepatica which live outside and in the duodenum of the mammalian host is switched to anaerobic respiration in the adult stage worms living in the bile duct [34,35]. As for E. multilocularis, a certain proportion of larval cysts in liver may well access the portal vein and hepatic vein that exhibit higher arterial oxygen tension in the intermediate hosts [36]. These larval cysts may actively use oxidative phosphorylation, as this pathway is more effective than fumurate respiration for producing ATP. However, the larval cysts grow within the liver, making it difficult for them to have access to oxygen. In this case, fumurate respiration might be the predominant respiratory system in accordance with the size and site of the larval cysts. Although the oxygen concentration that enables oxidative phosphorylation to serve as the predominant respiratory chain has not been investigated in this study, the efficacy of ATV in vivo study indicates that oxidative phosphorylation might be flexibly used by the larval cysts even at the lower oxygen concentration. On the other hand, A. suum and F. hepatica migrate to hypoxic sites in the host and completely change their respiratory chain from the aerobic to anaerobic when they become adults [14,35,37]. Bacteria change the respiratory chain according to the oxygen supply and this is mainly controlled by transcriptional regulators that detect oxygen and nitric oxide levels [38,39]. However, little is known about the molecular mechanism of oxygen adaptation in parasites [33].

One of the important observations of the present study is that E. multilocularis complex III could be a promising drug target. Mitochondrial complex III (cytochrome bc1 complex) consists of cytochrome b, cytochrome c1, and Rieske iron-sulfur protein. Complex III has two quinone binding sites, quinol oxidation site (Qo site) and quinone-reducing site (Qi site). Antimycin A specifically binds to the Qi site, while myxothiazol, azoxystrobin, and ATV specifically inhibit binding of quinones to the Qo site [40,41]. In the present study, we demonstrated biochemically that ATV inhibited E. multilocularis complex III at an IC50 of 1.5 nM. ATV inhibits P. falciparum complex III with an IC50 of 0.13–0.46 nM [42,43]. However, P. falciparum complex III is not involved in oxidative phosphorylation and ATP production, but rather in the biosynthetic pathway of pyrimidine in the red blood stage parasite [44–46]. Blocking the synthesis of pyrimidine by the inhibition of complex III with ATV leads to antiparasitic effects against P. falciparum. Babesia possesses oxidative phosphorylation and anaerobic respiratory systems [47]. Although the target of ATV against Babesia is mitochondrial complex III, ATV may have antiparasitic effects regardless of the alterations in oxygen concentration, because it inhibits dihydroorotate dehydrogenase directly, which is the fourth enzyme in the pyrimidine synthesis pathway in Babesia with an IC50 of 3 nM [48]. In contrast, oxidative phosphorylation is functional in T. gondii. ATV inhibits complex III of T. gondii with an IC50 of 0.03 μM and disrupts the mitochondrial membrane potential [49]. Thus, effect of ATV on the parasite mitochondria is quite diverse.

Interestingly, unlike other quinone binding site inhibitors, ATV is an inhibitor of not only complex III but also of complex II in E. multilocularis. ATV inhibits E. multilocularis complex II with an IC50 of 0.69 μM. However, the antiparasitic ability of ATV against E. multilocularis complex II is weak compared with that of atepin A5 with an IC50 of 42 nM, as protoscoleces were not eliminated by ATV under anaerobic conditions.

The in vivo experiments revealed that complex III of the larval cysts has potential as a chemotherapeutic target for the development of novel drugs. We showed a significant difference between the ATV group and control one in the sizes of the larval cysts after treatment, which suggests that complex III contributes to the growth of larval cysts in the intermediate host. However, the effect of ATV was significantly lower than that of ABZ in treated animals. As described above, although ATV is highly effective against larval cysts using oxidative phosphorylation, it may have a weak effect on those using fumurate respiration under anaerobic conditions. The larval cysts of E. multilocularis may flexibly adjust their respiratory chain to cope with their growth and alterations in oxygen concentration. In P. westermani, which uses both aerobic and anaerobic respiration in the mammalian lung by forming cysts; the proportion of specific activity of cytochrome c oxidase between P. westermani and mammalian system (bovine heart) was 0.43 [17].
However, the proportion of specific activity of cytochrome c oxidase between *E. multilocularis* and mammalian system (porcine heart) was 0.032. The difference between these results might indicate that a small portion of aerobic respiration likely functions in *E. multilocularis*, as suggested by the culture treatment and in vivo experiments. As a result, inhibition of both mitochondrial complexes II and III could lead to the strong anti-helminthic effects against larval cysts in vivo. Our goal is to combine ATV with a strong complex II inhibitor to block fumarate respiration under anaerobic conditions. We are currently examining clinically safe complex II inhibitors, including ascofuranone (AF) derivatives, to resolve this problem. In addition, as the biosynthetic pathway of AF has been clarified, thereby making its industrial-level production possible [50], a combination of ATF and AF derivatives would be a promising treatment of AE.

In the clinic, almost 50% of patients experience one or more side effects following ABZ administration, and 6.9% show severe liver toxicity requiring drug switches or pauses [51]. ATV is safe for long-term treatment of malaria in clinical practice. Experiment B in this study suggested that ATV might prove to be an important drug candidate for novel therapeutic approaches, including combination chemotherapy with complex II inhibitors and/or others.

**Funding**

This work was supported by KAKENHI of Japan Society for the Promotion of Science, grant number JP17K08815 and JP19H03436 to Hirokazu Kouguchi and Kiyoshi Kita, respectively, and Research Program on Emerging and Re-emerging Infectious Diseases (17k0108119j0001) to Kiyoshi Kita.

**Declaration of Competing Interest**

None.

**Acknowledgments**

We are indebted to the anonymous reviewers for providing insightful comments for revising our manuscript.

**References**


