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Medical Treatment of *Echinococcus multilocularis* and New Horizons for Drug Discovery: Characterization of Mitochondrial Complex II as a Potential Drug Target

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/intechopen.68565>

Abstract

As an efficient drug for alveolar echinococcosis (AE) is still not available, new chemotherapy targets are necessary. The mitochondrial respiratory chain may be a good drug candidate because parasite respiratory chains are quite different from those of mammalian hosts. For example, *Ascaris suum* possesses an NADH-fumarate reductase system (fumarate respiration) that is highly adapted to anaerobic environments such as the small intestine. It is composed of mitochondrial complex I (NADH-ubiquinone reductase), complex II (succinate-ubiquinone reductase), and rhodoquinone. We previously demonstrated that fumarate respiration is also essential in *E. multilocularis*. Quinazoline, a complex I inhibitor, inhibited growth of *E. multilocularis* larvae in vitro. These results indicate that fumarate respiration could be a target for *E. multilocularis* therapy. In the current chapter, we focused on complex II, which is another component of this system, because quinazoline exhibited strong toxicity to mammalian mitochondria. We evaluated the molecular and biochemical characterization of *E. multilocularis* complex II as a potential drug target. In addition, we found that ascofuranone, a trypanosome cyanide-insensitive alternative oxidase inhibitor, inhibited *E. multilocularis* complex II at the nanomolar order. Our findings demonstrate the potential development of targeted therapy against *Echinococcus* complex II.

Keywords: *Echinococcus multilocularis*, drug discovery, fumarate respiration, mitochondrial complex II, ascofuranone

1. Treatment and prevention of echinococcosis

1.1. Treatment of alveolar echinococcosis

Echinococcosis is a zoonosis caused by adult or larval stage *Echinococcus*, tiny cestode parasites in the family *Taeniidae*. The two major species of medical and public health importance are *Echinococcus granulosus* and *E. multilocularis*, which cause cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively. This chapter describes the main objective of AE therapy, which is more difficult to treat than CE. Treatment for CE generally includes albendazole, surgery, and puncture, aspiration, injection, and reaspiration (PAIR) therapy, or a combination thereof, according to the World Health Organization diagnostic classification [1, 2]. The cure rate of PAIR therapy is especially high, at 97% for cysts exceeding 5 cm in size [3, 4]. However, PAIR therapy has not been adopted for AE. In surgical treatment of AE, radical resection is required for hepatic lesions. Conservative and palliative surgery are not recommended since they have no advantage over chemotherapy [5]. Liver transplantation is a therapeutic option for patients unsuitable for radical surgery with hepatic failure. Extrahepatic spread of AE in liver transplant recipients may lead to a risk of relapse due to the use of immunosuppressive agents after surgery [6, 7]. In addition, it is not easy to perform liver transplantation in developing countries without advanced medical equipment and high health care costs. As radical surgery for advanced AE is more difficult than that for CE, chemotherapy plays a key role in treatment of AE. Albendazole, a benzimidazole anthelmintic, is primarily used in chemotherapy for AE. The 15-year survival of albendazole treatment is 53–80% in patients not treated by surgery, according to the condition of cysts [3, 8, 9]. Furthermore, two-thirds of patients experienced one or more side effects of albendazole, and the development of major side effects led to permanent discontinuation of albendazole in 3.8% of patients [10]. Praziquantel, which is expected to have a synergistic effect with albendazole, is insufficient for AE [11]. Although *in vivo* studies have evaluated the effectiveness of a calcium channel blocker, thymol, and novel compounds are reported as new chemotherapy, they have only a limited effect on AE [12–14]. A recent report revealed that nitazoxanide, an anticipated promising drug, had no effect on treatment of AE [15]. These findings emphasize the difficulty in developing an effective drug for AE. Additionally, there are no other treatment options for patients in whom albendazole chemotherapy failed and who have no indications for liver transplantation.

1.2. Current status of the development of a vaccine against echinococcosis

Vaccine targets for echinococcosis are either intermediate or definitive hosts or both. EG95 was identified as a candidate vaccine antigen for intermediate hosts of *E. granulosus* in 1998 [16, 17]. As intermediate hosts of CE are livestock, such as sheep, goat, and cattle, vaccination of intermediate hosts of CE would presumably lead to the reduction of economic loss and the effective control of CE in the life cycle. In a pilot field trial of the EG95 vaccine, vaccine introduction in a sheep farm led to a statistically significant reduction in the number and size of hydatid cysts compared to the control area where the vaccine was not applied. The prevalence of infection in the vaccinated area was reduced by 62% compared to the control area [18].

The gene product of *E. multilocularis*, EM95, is homologous to EG95 [19]. Mice immunized with the EM95 recombinant protein following challenge infection showed a significantly decreased number of cysts compared with control mice [19]. Furthermore, EMY162 antigen, which is also homologous to EG95, was identified in 2007 [20, 21]. Several candidate vaccine antigens based on homology to EM95 or EMY162 were subsequently reported [22, 23]. In addition, a transmembrane protein, tetraspanin (TSP), was identified as the antigen protein of an AE vaccine [24]. Protective effects of recombinant TSP against AE have been reported [25, 26]. However, it is difficult to apply a vaccination strategy to wild mice, which are the main intermediate hosts of AE. Although human AE is a serious parasitic disease, there has been little progress on application of these vaccines to humans since safety standards are exceptionally high.

Development of a vaccine for the definitive host dog is important because such a vaccine might contribute to a considerable reduction of human CE and AE in endemic areas. However, no effective vaccine candidate has been identified despite various trials. Although some oral recombinant vaccines showed high levels of protection against *E. granulosus* in dogs [27–29], these reports have been criticized in terms of their statistical analyses [30]. However, mucosal immunization with a parasite surface antigen, with cholera toxin subunit B as a carrier molecule, induced a protective response to *E. multilocularis* infection in dogs [31]. Immunized dogs infected five times with *E. multilocularis* remained capable of excluding adult worms after a 6-month interval [32, 33]. These results suggest the potential effectiveness of the mucosal vaccine against *E. multilocularis* in definitive hosts.

2. Mitochondrial respiratory chain as a drug target

2.1. NADH-fumarate reductase system (fumarate respiration)

Since the 1970s, when albendazole became available for clinical use, no new drugs for echinococcosis have been identified, as mentioned above. As an efficient drug for AE is still not available, new chemotherapy targets are necessary [34, 35]. Our group has focused on the mitochondrial respiratory chain, namely the NADH-fumarate reductase system, of parasites as a potential drug target. Parasitic helminthes possess an NADH-fumarate reductase system that is highly adapted to anaerobic conditions [36, 37]. The parasitic nematode *Ascaris suum* is a suitable model for biochemical studies of mitochondrial NADH-fumarate reductase systems because the body sizes of adult worms are easy to manipulate. We previously reported that the NADH-fumarate reductase system is a good target for the development of novel, selective anthelmintic compounds as modeled in *A. suum* [38, 39]. It is composed of complex I (NADH-quinone reductase, NQR), complex II (quinol-fumarate reductase, QFR), and a low-potential electron mediator, rhodoquinone (RQ). Low-potential RQ transfers the reducing equivalent of NADH via complex I to complex II, and succinate is ultimately produced by QFR activity of complex II. The merit of this system is ATP synthesis using the coupling site of complex I even in the absence of oxygen. QFR catalyzes the reduction of fumarate to succinate, while SQR (succinate-quinone reductase, used in mammalian systems) does the oxidation of succinate in the opposite direction (**Figure 1**). The NADH-fumarate reductase

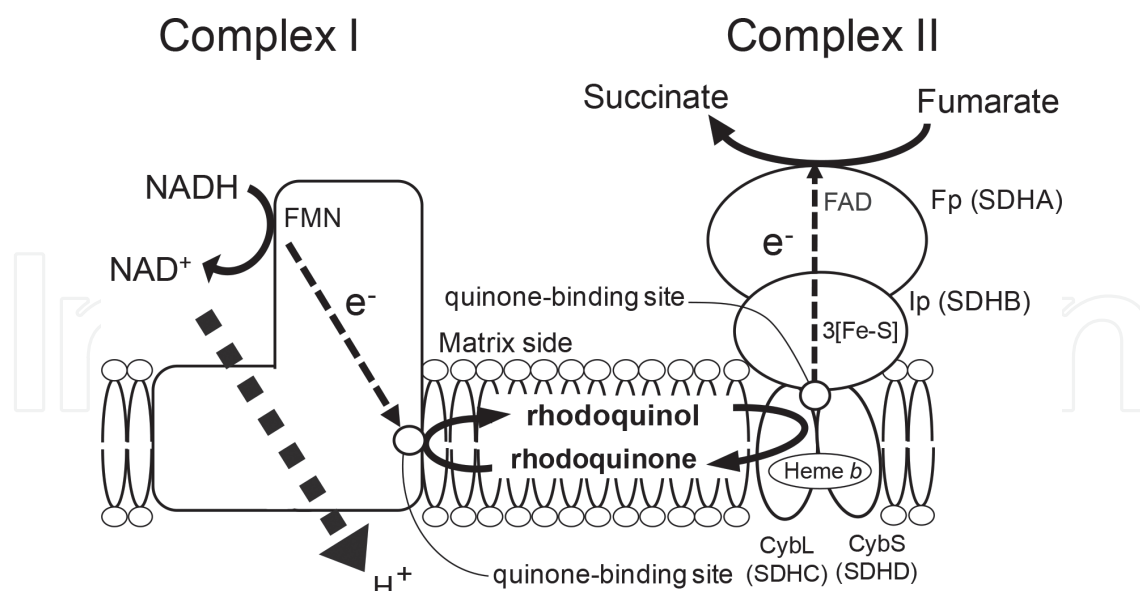


Figure 1. A schematic representation of the NADH-fumarate reductase system in adult *A. suum*, which catalyzes the last step of the phosphoenolpyruvate carboxykinase-succinate pathway. The NADH-fumarate reductase system is composed of complex I (NADH-quinone reductase), low-potential rhodoquinone (RQ), and complex II (quinol-fumarate reductase, QFR). In this system, electrons from NADH are transferred to RQ by the NADH-RQ reductase activity of mitochondrial complex I and then transferred to fumarate by the QFR activity of mitochondrial complex II through the quinone-binding site. Anaerobic electron transfer in complex I couples with proton transport across the mitochondrial inner membrane to generate ATP. *A. suum* complex II is composed of four subunits: flavoprotein subunit (Fp), iron-sulfur protein subunit (Ip), and cytochrome *b* large and small subunits (CybL and CybS, respectively). 3[Fe-S], iron-sulfur clusters; FAD, flavin adenine dinucleotide; and FMN, flavin mononucleotide.

system is absent in mammalian mitochondria living in aerobic conditions. Therefore, this unique respiratory system is considered to be a promising chemotherapeutic target for the development of novel anthelmintics.

2.2. Mitochondrial complex II

Complex II is a member of the tricarboxylic acid (TCA) cycle and respiratory chain. SQR as complex II catalyzes the oxidation of succinate to fumarate in the TCA cycle and transfers the electron to ubiquinone in the respiratory chain. QFR as complex II catalyzes the reduction of fumarate to succinate, a reverse reaction of succinate dehydrogenase (SDH), in the respiratory chain of mitochondria from anaerobic animals such as *A. suum* as described above. Generally, complex II consists of four subunits. Flavoprotein (Fp) subunit contains flavin adenine dinucleotide (FAD) as a prosthetic group and iron-sulfur protein (Ip) subunit contains three iron-sulfur (Fe-S) clusters [38]. The complex also contains large and small hydrophobic cytochrome *b* (CybL and CybS, respectively) subunits. The succinate-binding site is located in the Fp subunit, while the quinone-binding site is formed by the other three subunits, Ip, CybL, and CybS.

2.3. *E. multilocularis* complex II as a novel drug target

Our group has focused on the biochemical properties of complex II and its potential as a drug target against helminth infections [40–42]. This concept could be expanded to *Echinococcus*

species, which belongs to parasitic platyhelminthes, distinct from nematodes. As expected, we found that the NADH-fumarate reductase system played a dominant role in isolated mitochondria from larval *E. multilocularis* [36]. In addition, quinazoline, an inhibitor of complex I, exhibited antiechinococcal activity under in vitro culture conditions [36]. These findings suggest that the NADH-fumarate reductase system is a potential therapeutic target in *E. multilocularis*. However, it is difficult to synthesize quinazoline derivatives. In addition, quinazoline and its derivatives exhibited strong toxicity in mammalian cells. Therefore, we focused on mitochondrial complex II in the NADH-fumarate reductase system as a drug target. Flutolanil and atpenin A5 are known effective inhibitors of the quinone-binding site of *A. suum* complex II [42, 43]. Elucidation of crystal structures of *A. suum* complex II in the presence of flutolanil provided useful information for the structure-based design of a more effective inhibitor [39, 44]. As a crystallographic analysis of *Echinococcus* complex II is challenging, we conducted comparative analyses of *E. multilocularis* with other parasites and host enzymes.

We cloned cDNA of complex II and assembly factors of *E. multilocularis* and purified subunits of complex II from mitochondria by high resolution clear native electrophoresis (hrCNE) to determine N-terminal amino acid sequences of mature subunits. In addition, we investigated the effects of several quinone-binding site inhibitors on *E. multilocularis* complex II.

3. Characterization of *E. multilocularis* complex II as a drug target

3.1. Cloning and sequence analyses of the genes for four constitutive subunits and two assembly factors

Since genome project data from Brehm and colleagues were released in advance on their website (Wellcome Trust Sanger Institute: <http://www.sanger.ac.uk>), we first identified complex II-related genes by BLAST search using human and other eukaryotic sequences as queries. A partial or full open reading frame (ORF) of four subunits composing mature complex II and two assembly factors were identified by TBLASTN search against expressed sequence tag (EST) or genomic contig and shotgun reads. Two isoforms were found for the Ip subunit, and the other subunits were encoded in a single gene each. Primers for the coding region of each gene were designed based on this information (**Table 1**). First, the coding region of the seven genes was amplified by gene-specific PCR, and sequences were determined after insertion into a cloning vector. For rapid amplification of cDNA ends (RACE) of 5' and 3' ends, new primers were designed from the confirmed coding regions. Finally, cDNA sequences of *sdha* (Fp), *sdhb1* (Ip1), *sdhb2* (Ip2), *sdhc* (CybL), *sdhd* (CybS), *sdhaf1*, and *sdhaf2* were determined (DDBJ accession numbers: AB699145–AB699151).

During 3'RACE of *sdhaf1*, we found that the first PCR yielded an exceptionally long amplified DNA fragment (ca. 1.4 kbp) compared to the ORF of *sdhaf1* (288 bp). Interestingly, this fragment contained another ORF homologous (27% of amino acid identity) to Tam41p in budding yeast (GenBank ID: NP_011560) [45], whose start codon overlapped the stop codon of *sdhaf1*. 3'RACE was conducted again with gene-specific primers complementary to the tam41 coding region, which is closer to the 3'-terminal end of cDNA compared to the initial 3'RACE

Primer		Sequences	Experiment
Fp-1	F	5'-AGGCTCCCAAGGCTGTTATC-3'	ORF
Fp-2	R	5'-GCAGACGTTCTGATCTAAAG-3'	ORF & 5'RACE 1 st
Fp-3	F	5'-GCTTGCGCCCTCGATATT-3'	3'RACE 1 st
Fp-4	F	5'-GTCTCATATGGAACCTCGGAC-3'	3'RACE 2 nd
Fp-5	R	5'-CGGAGTGAGCGACCGTATAGAG-3'	5'RACE 2 nd
Fp-6	F	5'-CAGTTTTCTGTCACCTTCATGG-3'	ORF-full
Fp-7	R	5'-CCTTGAACGATTAGTAGGAACGG-3'	ORF-full
Ip1-1	F	5'-TCCGTTCTCTGCTTTTCGAC-3'	ORF & 3'RACE 1 st
Ip1-2	R	5'-TCAGCATCTTCTTGATCTCACC-3'	ORF
Ip1-3	F	5'-TCTGCGCTATTCCCAAAGAT-3'	3'RACE 2 nd
Ip1-4	R	5'-GGAATAGCGCAGAGACAGGCCAGAC-3'	5'RACE 1 st
Ip1-5	R	5'-GAGCGTCAAGCATCATGGGAC-3'	5'RACE 2 nd
Ip1-6	F	5'-TCGCCAGGAGAATGAATTCC-3'	ORF-full
Ip1-7	R	5'-GTCACCTCGAACCGGTTTCAG-3'	ORF-full
Ip2-1	F	5'-GATAACTTGCAAGCGTGC-3'	3'RACE 1 st
Ip2-2	F	5'-GTCCGGCTACATTGATACAC-3'	3'RACE 2 nd
Ip2-3	R	5'-AATTTTGGTGACTTTATTGCTCCTC-3'	5'RACE 1 st
Ip2-4	R	5'-AATGTGAGGGTTGGGTCGCAT-3'	5'RACE 2 nd
Ip2-5	F	5'-GTGGTGGAACATGAATTGTGTTTG-3'	ORF-full
Ip2-6	R	5'-GAATGCATCACAATGCCAGGA-3'	ORF-full
CybL-1	F	5'-TTTTTGCGAACGTTCTGTTG-3'	ORF & 3'RACE 1 st
CybL-2	R	5'-CTTCCACAGGTCCGAACAC-3'	ORF
CybL-3	F	5'-AAGGGCAGCACAAAGTGAGG-3'	3'RACE 2 nd
CybL-4	R	5'-CCATGGCGACACCAGTAGCACGG-3'	5'RACE 1 st
CybL-5	R	5'-AATATGTGAGGCGACCACGGG-3'	5'RACE 2 nd
CybL-6	F	5'-GCGCGGTTAGACATGTCTG-3'	ORF-full
CybL-7	R	5'-GGCTAGCAACATCTAGCTCCTG-3'	ORF-full
CybS-1	F	5'-ATGTCTTTCGCGCTTTTGG-3'	ORF
CybS-2	R	5'-TTTTGACGCCCTTAATAACACC-3'	ORF, 5'RACE 1 st & 2 nd
CybS-3	F	5'-GCGAAGTTGGGGACAGCT-3'	3'RACE 2 nd
CybS-4	F	5'-GGCTTCATTGGCAGGATGTC-3'	ORF-full
CybS-5	R	5'-CACTGCGTGCTCAAAGAGACC-3'	ORF-full
AF1-1	F	5'-ATGTCTCGCCCATATAGTCAACTTC-3'	ORF & 3'RACE 1 st
AF1-2	R	5'-TTAATCTTGTTTATGGGGAGGAAAAG-3'	ORF & 5'RACE 1 st
AF1-3	F	5'-GACAGGTTCTGAAGCTCTATAAGGA-3'	3'RACE 2 nd

Primer		Sequences	Experiment
AF1-4	F	5'-ATATGGGGTCGTTGGTTATG-3'	3'RACE 1 st
AF1-5	F	5'-AGTAGAGACGCCAATCACCACGA-3'	3'RACE2 nd
AF1-6	R	5'-GACCTGCCTCAAGGTCTTCC-3'	5'RACE 2 nd
AF1-7	F	5'-TTGATTATTAGCGCAATATAAGGTG-3'	ORF-full
AF1-8	R	5'-TTTTCTAAATGTTTTATTCAGGCAAA-3'	ORF-full
AF2-1	F	5'-ATGGTGCTGTCTCTTAATCGTTTGAC-3'	ORF & 3'RACE 1 st
AF2-2	R	5'-CTACACAGTGTGGGGGTGAATATTG-3'	ORF & 5'RACE 1 st
AF2-3	F	5'-TCTGTTGCGACAATGAGGAG-3'	3'RACE 2 nd
AF2-4	R	5'-TCTGTTGCGACAATGAGGAG-3'	5'RACE 2 nd
AF2-5	F	5'-ACTATTAAACTTTCTGCTTGGTTGCAT-3'	ORF-full
AF2-6	R	5'-GTTGTATATAATGCTGTAATTAATAAGGACAAC-3'	ORF-full

Table 1. Gene-specific primers used in this chapter.

experiment. The presence of this polycistronic mRNA was confirmed by PCR with primers that were complementary to the 5' untranslated region (UTR) of *sdhaf1* and 3' UTR of *tam41*.

The number of amino acids of all determined proteins and their sequence identities with corresponding proteins in *A. suum* and humans are summarized in **Table 2**. The position from the first methionine (Met) of the N-terminal sequences of the four subunits in mature complex II is also listed in parentheses next to these 10 amino acids' sequences in **Table 3**. The two isoforms of Ip share the same sequence in this region. Two isoforms of Ip were reported in the parasitic nematode *Haemonchus contortus* [46]. Additionally, there are two isoforms of Fp, type I and II, in human complex II. It is speculated that complex II with type II Fp has a higher QFR activity and plays an important role in fumarate respiration in human mitochondria as the terminal oxidase of the system [38]. Isoforms of *E. multilocularis* Ip might be related to

Gene	Product	Amino acids	Identity of amino acid sequence (%)	
			A. suum (adult)	Human
<i>sdha</i>	Fp	647	68	72
<i>sdhb1</i>	Ip1	282	58	62
<i>sdhb2</i>	Ip2	282	57	62
<i>sdhc</i>	CybL	194	23	31
<i>sdhd</i>	CybS	153	27	30
<i>sdhaf1</i>	SDHAF1	95	27	39
<i>sdhaf2</i>	SDHAF2	140	31	41

Table 2. Summary of cloning and translated amino acid sequence.

Gene	Product	Amino acid of premature protein (upper)
		N-terminal amino acid of mature protein (positions*)
<i>sdha</i>	Fp	MAFLVRASFASFAARLGCLPTFAGASRHVSTVGKDYTI VSTVGKDYTI (29–38)
<i>sdhb1</i>	Ip1	MNSVLCFSTRYACVIGQTARYASTGPVMKKF ASTGPVMKKF (22–31)
<i>sdhb2</i>	Ip2	MNCVCSLSLRFEFLLIQTARYASTGPVMKKF ASTGPVMKKF (22–31)
<i>sdhc</i>	CybL	MSVFANVLLRAHAAPFRGVAARNLSMALQPLLRTAPVLSATKHYKGSTSEEVRL KGSTSEEVRL (46–55)
<i>sdhd</i>	CybS	MSFALLASKHLIRRAAVSSFVSANACRTLVTNNKAKLGTAPQPV AKLGTAPQPV (37–46)

*Positions of the N-terminal 10 amino acids from the first Met in cDNA.

Table 3. N-terminal amino acid.

changes in the respiration system, although the expression ratio of the two isoforms must be estimated during the life cycle.

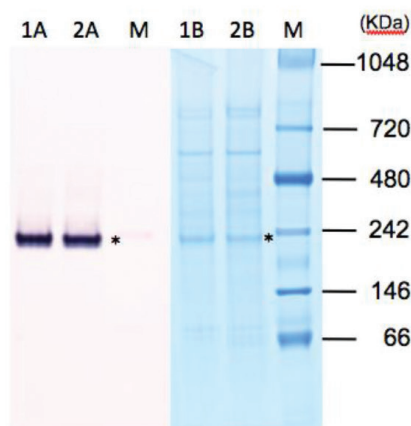
3.2. Purification of *E. multilocularis* complex II by electrophoresis

As all the genes for mitochondrial complex II are chromosomally encoded and the N-terminal of each peptide is processed during mitochondrial localization, *E. multilocularis* complex II was partially purified and N-terminal amino acid sequences of the subunits were determined. Mitochondrial samples solubilized and separated by hrCNE were further subjected to two-dimensional SDS-PAGE (see Section 5). Since first-dimensional electrophoresis was conducted by hrCNE, the four subunits of complex II were expected to align under the SDH activity stained band (**Figure 2A**). Based on the apparent molecular weight, candidates of the four subunits were selected (indicated by black arrows): Fp (75 kDa), Ip (28 kDa), CybL (15 kDa), and CybS (12 kDa) (**Figure 2B**). Bands corresponding to these subunits in protoscol- eces (larval stage) and adult *E. multilocularis* were detected at the same positions. From the partially purified sample, four subunits could be stained by coomassie brilliant blue (CBB), and N-terminal amino acid sequences were determined in 10 residues for those four bands (**Table 3**).

3.3. Inhibition of complex II by flutolanil, atpenin A5, and ascofuranone

We developed a method to separate *E. multilocularis* mitochondria to study its biochemical properties, including inhibitor screening. We constructed a quinone-binding site inhibitor library since this site is considered a good target for antiparasitic drugs. The inhibitory effect of flutolanil, atpenin A5, and ascofuranone as representative inhibitors was subsequently analyzed.

SDH activity staining and CBB stain



2D SDS-PAGE

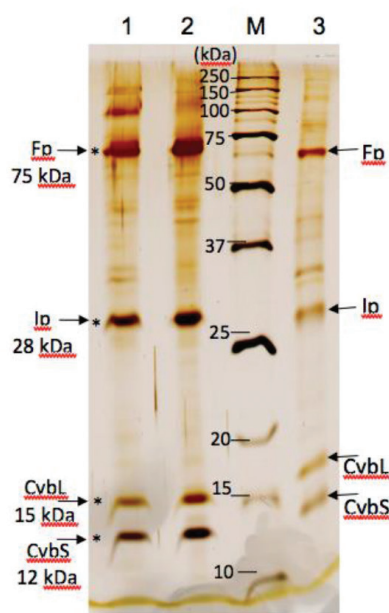


Figure 2. A SDH (succinate dehydrogenase) activity staining. Lane 1A: adult *E. multilocularis* and lane 2A: protoscoleces (PSC). CBB (Coomassie Brilliant Blue) staining. Lane 1B: adult *E. multilocularis* and lane 2B: PSC. Asterisks indicate complex II. Lane M: molecular weight markers. B. 2D SDS-PAGE. Comparison of (1) PSC complex II, (2) adult complex II, (3) purified *A. suum* complex II, and (M) molecular weight markers. Candidates of four subunits were selected (indicated by black arrows), which correspond to flavoprotein (Fp, 75 kDa), iron-sulfur cluster protein (Ip, 28 kDa), cytochrome *b* large subunit (Cvbl, 15 kDa), and cytochrome *b* small subunits (Cvbs, 12 kDa). Asterisks indicate PSC complex II subunits. PSC and adult complex II subunits demonstrated similar sizes.

The 50% inhibitory concentration (IC_{50}) values of flutolanil, atpenin A5, and ascofuranone for *E. multilocularis* QFR are shown in **Table 4**. The selectivity index was expressed with the IC_{50} of each inhibitor for porcine SQR. The IC_{50} and selectivity index of flutolanil for *E. multilocularis* QFR were 15 μ M and 2.9, respectively (IC_{50} of porcine SQR was 44 μ M), while the IC_{50} and selectivity index of flutolanil for *A. suum* QFR were 0.058 μ M and 758, respectively [44]. The IC_{50} of atpenin A5 for porcine SQR (0.0036 μ M) was lower than that for *E. multilocularis* (0.059 μ M) and *A. suum* QFR (0.012 μ M) [43]. Surprisingly, the IC_{50} and selectivity index of ascofuranone,

Inhibitor	IC ₅₀ (μM)			Selectivity index
	<i>E. multilocularis</i> QFR	<i>A. suum</i> QFR	Porcine SQR	Porcin IC ₅₀ / <i>E. multilocularis</i> IC ₅₀
Flutolanil	15 ± 0.28	0.058*	44	2.9
Atpenin A5	0.059 ± 0.0063	0.012**	0.0036	0.061
Ascofuranone	0.85 ± 0.070	10	300	350

*Harada et al. Biochimica et Biophysica Acta. 2013;1827:658-667.
**Miyadera et al. Proceedings of the National Academy of the Sciences USA. 2003;21:473-477.

Table 4. The inhibitory effect of representative quinone-binding site inhibitors.

which was developed as an antitrypanosomal drug, for *E. multilocularis* QFR, were 0.85 μM and 350, respectively, although the IC₅₀ of ascofuranone for *A. suum* QFR was 10 μM.

4. Discussion

4.1. Features of *E. multilocularis* complex II

In this chapter, the molecular characterization of *E. multilocularis* complex II was performed, including complex II inhibitor screening.

The FAD prosthetic group of Fp is localized in the FAD-binding domain by a covalent bond to histidine (His) and hydrogen bonds with highly conserved residues across amino acid sequences of complex II from various species [44, 47]. Fp in *E. multilocularis* has this conserved sequence, including the segment containing FAD-bound His.

The Ip subunit generally contains three Fe-S clusters coordinated by cysteine (Cys) residues [10]. The Ip subunit of *E. multilocularis* also has three well-conserved Cys-rich regions associated with the Fe-S cluster. An unusual amino acid substitution was found in the Fe-S cluster related to the Cys-rich region. A comparison of this amino acid with the known crystal structure of complex II placed it spatially in the vicinity of the 4Fe-4S center. We found two isoforms of *E. multilocularis* Ip (EmIp1 and EmIp2), which differ from each other by remarkable one amino acid residue. Leucine (Leu) 180 of EmIp1 and phenylalanine (Phe) 180 of EmIp2 are very rare substitutions in the second conserved Cys-rich region among complex II. This position is primarily an alanine (Ala), and infrequently a glycine, in bacterial or eukaryotic enzymes. Ala is generally located adjacent to the second conserved Cys in the second Cys-rich region of Ip, interacting with the Fe-S cluster in many species. *E. multilocularis* complex II functions as QFR with RQ as the electron donor. Interestingly, *Rhodospirillum rubrum*, a photosynthetic bacterium that we consider as a candidate expression host for *E. multilocularis* complex II, has a Phe at this position, similar to EmIp2. Because *R. rubrum* has RQ and ubiquinone as hydrophobic electron carriers in the cytoplasmic membrane, its complex II may function as QFR, utilizing RQ similar to *E. multilocularis*. Cloning of *sdhb* (Ip) highlighted the features of *E. multilocularis* complex II. The role of this amino acid residue in the catalytic activity will be studied in future mutational analyses of *E. multilocularis* and *R. rubrum* complex II.

In our chapter, mitochondrial complex II of *E. multilocularis* was purified by preparative hrCNE, and N-terminal amino acid sequences of all four subunits from mature enzymes were determined. The benefit of employing preparative hrCNE is that the loss of protein during the experiment is small since the condition is already fixed by analysis on a minigel. Purification starting with 2.6 mg of mitochondrial protein successfully yielded a sufficient amount of purified complex II for subsequent protein sequencing analysis.

4.2. Identification of *sdhaf1* and *sdhaf2*

Functional expression of eukaryotic complex II in other organisms is difficult because complex II is a multi-subunit enzyme, and many prosthetic groups, such as FAD, are associated with this enzyme. In this condition, two important proteins, succinate dehydrogenase assembly factor 1 (*SDHAF1*) and *SDHAF2* (*SDHAF2*, or *SDH5*), involved in the synthesis of functional complex II were reported. Their gene products are not the components of mature complex II [48–50]. *SDHAF1* is suggested to play an essential role in complex II assembly. *SDHAF2* may be required for the insertion of FAD cofactor into Fp. Coexpression of these genes with the four subunits present in mature complex II may be required for functional expression of eukaryotic complex II in heterologous expression systems such as bacteria.

Although *sdhaf2* of *A. suum* and *Caenorhabditis elegans* was annotated, *sdhaf1* has not been annotated in the EST database [<https://www.ncbi.nlm.nih.gov/nucest>] or WormBase [<http://www.wormbase.org>]. Furthermore, the genome annotation of *sdhaf1* has not been assigned in the draft genome sequence and transcriptome analysis of *Echinococcus* [51]. TBLASTN search using human and other eukaryotic sequences as queries in WormBase [http://www.wormbase.org/tools/blast_blat] against the genome detected the *sdhaf1* candidate region as an intron-less structure on chromosome V, corresponding to positions 4710485–4710739 (genomic position: 4710485–4710739). In the EST database, the corresponding sequence was found as an ORF before the start codon of *cif-1*, an ortholog of human eukaryotic translation initiation factor 3, and ORF in several mRNA variants of *cif-1* (e.g., yk1259f02.5). However, *cif-1* itself is not related to complex II or Tam41P. BLASTP search in WormBase did not yield the sequence found in the genome and EST. Taken together, *sdhaf1* found in *C. elegans* genome and its expression was observed, although not annotated. A homologous gene was also found in *A. suum* (GenBank ID: J1213553). After the first report of *SDHAF1* and *SDHAF2*, several studies have reported *SDHAF2* but not *SDHAF1*. Identification of the prokaryotic gene corresponding to *sdhaf2*, named *sdhE* in prokaryotes, suggests a common role of this gene for the assembly process of the complex II. However, *sdhaf1* was not annotated as a protein coding gene in WormBase, which is one of the best organized and updated genes and protein databases for *C. elegans*. Cloning of *E. multilocularis sdhaf1* revealed polycistronic expression of this gene with downstream tam41. The same genome structure was conserved in *Schistosoma mansoni*, the first genomic reference for Platyhelminthes. A homologous ORF was identified in *C. elegans* EST database as a part of *cif-1* mRNA. Considering the small size of this gene (<300 bp) and the gene arrangement in *E. multilocularis* and *S. mansoni*, transcription in a polycistronic manner in *C. elegans* does not necessary indicate a correlation between *sdhaf1* and *cif-1*. However, because of its small size and mRNA structure, this gene might have been overlooked in this organism.

4.3. Inhibition of complex II by a quinone-binding site inhibitor

An inhibitor of the mitochondrial respiratory chain, atovaquone, has been used as an antimalarial agent [52]. Thus, it is reasonable to identify potent and specific inhibitors for the respiratory chain of *E. multilocularis*. Flutolanil, a commercially available fungicide, specifically inhibits helminth complex II [42]. The IC₅₀ and selectivity index of flutolanil for *A. suum* were 58 nM and 762, respectively. The flutolanil-binding site is located at the RQ-binding pocket, which is formed at the interface domain composed of three subunits, Ip, CybL, and CybS [39, 44]. Moreover, CH- π interaction between flutolanil isopropyl group and tryptophan69 (Trp) in CybL is one of the significant factors for the highly specific inhibitory effects of flutolanil against *A. suum* complex II [44]. Our finding that Trp69 is replaced with methionine (Met) in *E. multilocularis* and human (arrow A in **Figure 3**) likely explains why complex II activity in *E. multilocularis* was not inhibited by flutolanil. In fact, Harada et al. reported that porcine complex II is resistant to flutolanil since Trp69 is replaced by Met in porcine CybL [44]. Furthermore, in *E. multilocularis*, a phenylalanine (Phe) is located four amino acids downstream of the Met (arrow B in **Figure 3**). This Phe is replaced with isoleucine in human. This information is useful for the design of selective inhibitors of *E. multilocularis* complex II because the Phe residue forms a strong interaction, such as CH- π or cation- π interaction, with the inhibitor [53, 54].

			(A)	(B)	
			↓	↓	
<i>E. m</i>	CybL	NIKLRPWSPHILIYSPPLCMRNSFLHRATG			98
Hum	CybL	NIGSNRPLSPHITIIYSWSLPMAMSTCHRGTTG			75
<i>A. s</i>	CybL	QRALKRPIAPHLTIYKPQMTIMVSGLHRVTG			79

Figure 3. A comparison of the amino acid sequence of cytochrome *b* large subunit (CybL) from *E. multilocularis* (*E. m*), human (Hum), and *A. suum* (*A. s*). The arrow A indicates that tryptophan at position 69 is replaced with methionine in *E. multilocularis* and human. The arrow B indicates the phenylalanine four amino acids downstream of the methionine at arrow A in *E. multilocularis* is changed to isoleucine in human.

Interestingly, ascofuranone, which is a specific quinone-binding site inhibitor of cyanide-insensitive trypanosome alternative oxidase in *Trypanosoma brucei* mitochondria [55, 56], inhibited *E. multilocularis* QRF at the nanomolar order. To our knowledge, this study is the first to demonstrate that ascofuranone acts as a complex II inhibitor. Furthermore, our preliminary study showed ascofuranone has the ability to kill protozoa in culture within several days (data not shown). Our findings may aid in the development of new targeted therapy against *Echinococcus* complex II. We have synthesized more than 400 ascofuranone derivatives and will evaluate the structure-activity relationship in in vitro and in vivo studies.

5. Experimental information

5.1. Isolation of *E. multilocularis* protoscoleces and preparation of enriched mitochondrial fractions

The Nemuro strain of *E. multilocularis*, which is maintained at biosafety level 3 in the Hokkaido Institute of Public Health (Sapporo, Japan), was used in this study. The mitochondria

of protoscoleces were prepared as described previously [36]. To isolate protoscoleces, the mature larval parasites were minced with scissors, pushed through a metal mesh, and washed repeatedly with physiological saline. The isolated protoscolex sediment was suspended in 5 volumes of 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. The parasite materials were homogenized with a motor-driven glass/glass homogenizer. The homogenate was diluted with the mitochondrial preparation buffer to 10 times the volume of the original protoscolex sediment and then centrifuged at $800 \times g$ for 10 min to precipitate cell debris and nuclei. The supernatant was then centrifuged at $8000 \times g$ for 10 min to obtain the mitochondrial pellet. The pellet was resuspended in mitochondrial preparation buffer (without malonate) and centrifuged at $12,000 \times g$ for 10 min. The enriched mitochondrial fraction was suspended in mitochondrial preparation buffer (without malonate) [36].

5.2. hrCNE and two-dimensional SDS-PAGE

Separation profile of complex II by hrCNE was analyzed and the condition for purification was optimized by isocratic acrylamide minigel. The mitochondrial membrane of *E. multilocularis* was suspended as 10 mg/mL protein in gel buffer (50 mM Tris-HCl, pH 8.0). The membrane was solubilized by the addition of 50 mM Tris-HCl (pH 8.0), 4% SML (sucrose monolaurate), 40% (v/v) glycerol, and 2 mM sodium malonate on ice followed by centrifugation ($200,000 \times g$, 4°C , 30 min). The supernatant was mixed with 1/10 volume of loading dye (50% glycerol and 0.1% Ponceau S), and 55 μL of the resulting solution was applied to the well. Anode buffer ($20 \times$ running buffer) and cathode buffer ($20 \times$ running buffer, 0.02% n-dodecyl- β -D-maltoside, and 0.05% sodium deoxycholate) were used irrespective of the detergent for solubilization. Electrophoresis was performed in a cold room (4°C), starting with 100 and 250 V constant voltage for 1 h. The complex II band was visualized by GelCode Blue Safe Protein Stain (Thermo Scientific) or SDH activity staining. For activity staining, a gel strip was soaked in 10 mL of 5 mM Tris-HCl (pH 7.4) containing 25 mg of nitro blue tetrazolium, and then the reaction was started by the addition of 150 μL of 4 mg/mL phenazine methosulfate and 200 μL of 1 M sodium succinate. The complex II band was detected by activity staining and cut from the one-dimensional gel (CBB staining). The gel was equilibrated with SDS-PAGE buffer and then loaded onto the two-dimensional gel (4.5% acrylamide, 0.12% bisacrylamide, 0.25 M Tris-HCl (pH 6.8), 0.4% sodium dodecyl sulfate, 0.05% ammonium persulfate, and *N,N,N',N'*-tetramethylethylenediamine). Protein spots were visualized by silver staining (2D-Silver Stain-II kit, Cosmo Bio).

5.3. Partial purification of complex II by preparative hrCNE

Prepforesis® (ATTO) was used for preparative hrCNE. Separation gel (8% acrylamide) was prepared in a 1.6-cm diameter column at 2-cm height, and sample gel (3% acrylamide) was layered at 1-cm height. For solubilization, 2.6-mg protein of *E. multilocularis* mitochondria was precipitated, resuspended in 300 μL of solubilization buffer (10 mM Tris-HCl, 0.5 M 6-aminohexanoic acid, 5% (v/v) glycerol, 2.5% digitonin, pH 8.0), and kept on ice for 1 h. The resulting suspension was centrifuged ($200,000 \times g$, 10 min), and 3 μL of loading dye was added to the supernatant. The buffer system was the same as that of the minigel and kept at 6°C by a circulator. Electrophoresis was performed at 10 mA. The volume of one fraction was approximately 650 μL and 50 fractions were collected.

5.4. N-terminal amino acid determination of complex II constitutive subunits

Complex II-containing fractions from hrCNE were individually concentrated to approximately 70 μ L by Amicon Ultra-4 Centrifugal Filter Units (molecular weight cutoff is 50,000). After rough estimation of the concentration and purity of complex II on SDS-PAGE with silver staining, protein was precipitated with trichloroacetic acid. Briefly, 30 μ L of water and 10 μ L of 100% (w/v) trichloroacetic acid solution were added to 60 μ L of concentrated fraction and then the mixture was incubated on ice for 15 min and sedimented ($14,000 \times g$ for 10 min). These samples were then subjected to 12.5% SDS-PAGE, and proteins were transferred to an Immobilon-P membrane (Millipore), followed by staining with CBB G-250. Ten amino acid residues were determined with a Procise 494 cLC Protein Sequencing System (Applied Biosystems) at APRO Life Science Institute (Tokushima, Japan).

5.5. cDNA synthesis and cloning of complex II-related genes

Frozen mature larval parasites were pulverized with a mortar and pestle in liquid nitrogen, and total RNA was prepared using TRIzol LS Reagent (Invitrogen), according to the manufacturer's protocols, followed by further purification with RNeasy (Qiagen) and DNase I treatment. For 5'RACE, cDNA was synthesized with the SMARTTM RACE cDNA Amplification Kit (Clontech) using ReverTra Ace (Toyobo) as a reverse transcriptase. For 3'RACE, the oligo(dT) primer 5'-GACTCGAGTCGACATCGA(T)₁₇-3' was used for cDNA synthesis.

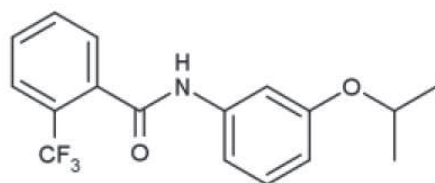
Primer sets to amplify the partial coding region of each subunit (except *sdhb2*) were designed based on the TBLASTN search, which was performed against the database of *E. multilocularis* EST or genomic contig and shotgun reads [<http://www.sanger.ac.uk/cgi-bin/blast/submitblast/Echinococcus>]. PCR was performed using Takara Ex Taq (Takara Bio) or PfuUltra II Fusion HS DNA polymerase (Stratagene). Gene-specific primers for 5'RACE and 3'RACE were designed from determined sequences in the coding region. For *sdhb2*, gene-specific primers were designed from the genomic contig (**Table 1**). All RACE experiments were performed as first PCR and nested PCR to obtain sufficient amplification of the DNA fragments. The universal primers for the first PCR and nested PCR for 5'RACE were universal primer mix and nested universal primers, respectively, which were provided within the SMARTTM RACE cDNA Amplification Kit. For 3'RACE, the adaptor primer 5'-GACTCGAGTCGACATCG-3' was used for both first and nested PCRs as the universal primer. These products were separated by electrophoresis on agarose gel, and target products were extracted with the MagExtractor-PCR & Gel Clean up kit (Toyobo). The gel-purified products were inserted into the pGEM-T vector (Promega) after A-tailing with Taq polymerase (Invitrogen) for sequencing. Entire ORFs were amplified using primers complementary to the determined 5' UTR and 3' UTR, and sequences were confirmed.

5.6. Enzyme inhibition assays

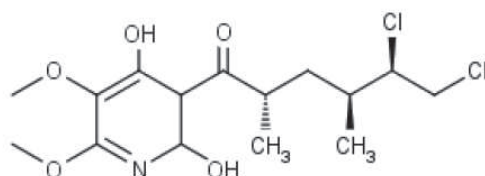
QFR and SQR assays were performed as described previously [36]. The final mitochondrial protein concentration was 50 μ g/mL of the reaction mixture. QFR and SQR activities were

assayed under anaerobic and aerobic conditions, respectively. QFR and SQR activities were determined by monitoring the absorbance change of decyl RQ (60 μ M) at 340 nm and ubiquinone-2 (60 μ M) at 278 nm (using SHIMADZU spectrophotometer UV-3000), respectively. We determined IC_{50} values of the quinone-binding site inhibitors against QFR activity of the mitochondria of protoscolec. Flutolanil (Wako), atpenin A5 (ENZO Life Sciences), and ascofuranone were used in the assays (**Figure 4**). Ascofuranone was obtained from Align Pharmaceutical.

A Flutolanil



B Atpenin A5



C Ascofuranone

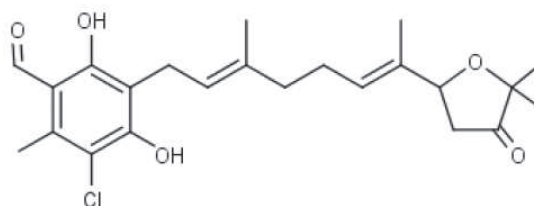


Figure 4. The chemical structure of inhibitors of the quinone-binding site. A. Flutolanil, a competitive inhibitor of the quinone-binding site of *A. suum* complex II. B. Atpenin A5, a competitive inhibitor of the quinone-binding site of complex II of many species. C. Ascofuranone, a potent inhibitor of cyanide-insensitive alternative oxidase of *Trypanosoma brucei*.

Acknowledgements

This work was supported in part by a grant-in-aid for Infectious Disease Control from the Science and Technology Research Partnership for Sustainable Development (no. 10000284 to KK), a grant-in-aid for the Bilateral Joint Research Project from the Japan Society for the Promotion of Science (no. 16035611-000722 to KK), and a grant from the Program for the Promotion of Basic and Applied Research for Innovations in Bio-Oriented Industries (to KK).

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