THE CHARLES UNIVERSITY IN PRAGUE, CZECH REPUBLIC
1ST FACULTY OF MEDICINE

DOCTORAL STUDY OF BIOMEDICINE

# MOLECULAR AND BIOCHEMICAL FEATURES OF THREE ENZYMES THAT MAY SERVE AS NEW DRUG TARGETS IN *Cryptosporidium parvum*

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SUMMARY OF Ph.D. THESIS (THESES)

2008

**DOCTORAL STUDY OF BIOMEDICINE**

**THE CHARLES UNIVERSITY IN PRAGUE AND THE ACADEMY OF SCIENCES, CZECH REPUBLIC**

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**The summary of the Ph.D. thesis was distributed (day) :**.....

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**Ph.D. dissertation defence (date, hour, place):** ……....

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**ABSTRACT**

**Molecular and biochemical features of three enzymes that may serve as new drug targets in *Cryptosporidium parvum***

*Cryptosporidium parvum* is a unicellular, obligatory and intracellular parasite belonging to the Phylum Apicomplexa. This parasite can infect both humans and animals, causing an acute diarrhea in immunocompetent persons, and a chronic life threatening infection in immunocompromised individuals. Although many drugs, to combat this parasite, have been empirically tested, there is no completely effective therapy to treat cryptosporidiosis in humans or animals. In recent years, the completion of the genome sequencing projects in *C. parvum* and *C. hominis* along with advances in molecular methods have significantly helped to increase our general understanding of the *C. parvum* metabolic machinery. However, our knowledge concerning many specific pathways and enzymes in *Cryptosporidium* is still limited. Their better understanding in this organism would aid in experimentation of new drugs and new strategy development to treat cryptosporidiosis in humans and animals.

In this dissertation, three essential metabolic enzymes of *C. parvum* have been tested:

**Pyruvate:NADP$^+$ oxidoreductase** (PNO) is a unique, core metabolic enzyme, responsible for converting pyruvate to acetyl-CoA. It is a rare fusion of an N-terminal pyruvate:ferredoxin oxidoreductase domain and a C-terminal cytochrome P-450 reductase domain. PNO is absent in human and animals and it is an essential enzyme for *C. parvum.* This protein was found to target the mitochondria in the distantly related protist *Euglena gracilis*. The determination of PNO subcellular localization either in the cytosol or in the relict mitochondrion of *C. parvum* is critical in assigning the biological role of this enzyme in the parasite therefore, its localization was determined.

***CpNARF*** is a *NARF*-like gene. The name NARF was derivated from human NARF - Nuclear prelamin A recognition factor that shares limited sequence similarity with iron-only hydrogenases but not their hydrogenase activity. The gene from *C. parvum* was cloned and characterized. Domain structure and phylogenetic analyses surprisingly revealed that *CpNARF* resembles more *NARF*-like genes from aerobic protists and higher eukaryotes rather than [Fe]-hydrogenases from other anaerobic protists and bacteria.

**S-adenosylhomocysteine hydrolase** is an enzyme regulating the S-adenosylhomocysteine metabolic pathway and the regulator of biological transmethylation reactions, which has been considered important in the target-based drug design of antiviral and antiparasitic drugs. Even though, this enzyme is present in human and animal cells, cryptosporidial enzyme differs from them having a plant-like insertion. Therefore, recombinant enzyme was used to evaluate the efficiency of potential inhibitors, leading to studies for elucidation their effect on the *in vitro* growth of *C. parvum*.

**ABSTRAKT**

**Molekulárně biochemické vlastnosti tří enzymů, které mohou sloužit jako nové cílové struktury pro navrhování léčiv proti *Cryptosporidium parvum***

*Cryptosporidium parvum* je jednobuněčný, obligatorní a vnitrobuněčný parazit patřící do kmenu Apicomplexa, který může infikovat hospodářská zvířata a člověka. Způsobuje akutní průjem u zdravých, imunokompetentních jedinců, ale může vést až k životu ohrožující, chronické infekci u imunokompromitovaných pacientů. Ačkoliv již bylo testováno mnoho potenciálních léčiv proti kryptosporiidu, zatím nebyla nalezena dostatečně účinná léčba. V posledních letech se výrazně prohloubily znalosti metabolických mechanismů tohoto parazita, a to hlavně díky výrazným pokrokům v molekulární biologii a osekvenování genomů *C. parvum* a *C. hominis*. I přesto musíme říct, že naše celkové porozumnění specifických metabolických drah je stále limitováno. Jejich detailní charakteristika nám může pomoci přiblížit se k nalezení nových cílových struktur a strategií pro vývoj účinějších léků proti lidské i zvířecí kryptosporidióze.

V této dizertační práci byly testovány tři nepostradatelné enzymy *C. parvum*:

**Pyruvát: $NADP^+$ oxidoreduktáza** (PNO) je unikátní enzym centrálního metabolismu, zodpovědný za přeměnu pyruvátu na acetyl-CoA. Jedná se o ojediněle se vykytující fúzi dvou domén, N-terminální pyruvátferedoxin oxidoreduktázy a C-terminální cytochrom P-450 reduktázy. Tento enzym se nevyskytuje u člověka a jiných mnohobuněčných organismů, charakterizovaný byl pouze u *Cryptosporidium parvum* a *Euglena gracilis*, kde se nachází v mitochondrii. Jeho lokalizace v kryptosporidiu nebyla známá. Umístění PNO v buňce ovlivňuje jeho biologickou funkci, proto byla ověřena možnost zda by se mohl nacházet v mitochondrii kryptosporidií nebo v jejich cytosolu.

***CpNARF*** je gen, jehož jméno bylo odvozeno od enzymu NARF (Nuclear prelamin A recognition factor), který se vyskytuje v jádře lidských buněk. NARF proteiny vykazují určitou sekvenční podobnost s [Fe] hydrogenázami, ale nemají jejich hydrogenázovou aktivitu. Kryptosporidiový gen byl zaklonován a charakterizován. Jeho doménová struktura a fylogenetická analýza překvapivě odhalila, že předpokládaný kryptosporidiový protein sdílí větší podobnost s NARF-like geny aerobních prvoků a vyšších eukaryotických organismů než s [Fe] hydrogenázami anaerobních prvoků a bakterií.

**S-adenosylhomocystein hydroláza** je enzym regulující S-adenosylhomocysteinovou metabolickou dráhu a současně regulátor transmetylačních reakcí. Je považován za důležitou cílovou strukturu pro navrhování antivirálních a antiparazitárních léků. I když je přítomen v lidských a savčích buňkách, liší se od nich tento kryptosporidiální enzym svojí rostlinou inzercí. Proto byl vytvořen re-kombinantní enzym, který byl charakterizován a dále testován v přítomnosti potenciálních inhibitorů. Současně byla zjišťována účinnost těchto inhibitorů na růst *C. parvum* v lidských buňkách *in vitro*.

## INTRODUCTION

*Cryptosporidium* is unicellular pathogen with a worldwide distribution. It has spread to all continents. It is present virtually everywhere (Caccio *et al*., 2005). Its host specificity is quite wide in contrast to other coccidia. It can be transmitted by water, food, animals and by person-to-person contact (Hellard *et al.*, 2003; MacKenzie *et al.*, 1995). Only 10 oocysts are sufficient to cause diarrheal infection in immunocompetent humans (Chappell *et al.*, 2006). Unlike other apicomplexans, the oocysts do not require external maturation and are excreted in very large numbers (O'Handley *et al.*, 1999). In contrast to other pathogens, the *Cryptosporidium* thick-walled oocysts are extremely resistant to common disinfectants, antiseptics and are problematic to kill even by chlorination which causes problems in water industry. Thin-walled oocysts are also produced, that are not excreted, but they rather autoinfect the host (Carrey *et al.,* 2004).

Even though *Cryptosporidium* belongs to the class coccidia, it differs from them in many features. These features are so important and there are so many of them, that some authors suggested to withdraw the *Cryptosporidium* species from the class of coccidia and propose a new class just for *Cryptosporidium* (Barta and Thompson, 2006; Slapeta, 2007). The outcome is: *Cryptosporidium* does almost not respond to commonly used anticoccidial drugs against its related parasites. The unresponsiveness of *Cryptosporidium* to anticoccidial drugs can be explained partly by its unique intracellular, but extracytoplasmic niche in the host cell, which may prevent drug delivery and uptake from the systemic circulation of blood (Thompson et al., 2005). However, the main reason is genetics. Many well-defined drug targets used for drug design in other apicomplexans (e.g. against coccidia) are either absent or highly divergent in *C. parvum*. This became more evident after the elucidation of both the *C. parvum* (Abrahamsen et al., 2004) and *C. hominis* (Xu et al., 2004) genomes. *Cryptosporidium* has adapted an extreme parasitic lifestyle. During the evolutionary process this parasite has lost most *de novo* synthetic abilities, and has maximized the number of biosynthetic molecules that could be salvaged from its host by its numerous transporters (e.g. sugars, nucleotides, amino acids) (Abrahamsen et al., 2004; Xu et al., 2004). In contrast to all other Apicomplexa, *Cryptosporidium* spared the whole apicoplast and has lost all apicoplast genes except those that were transferred to its genome prior to the physical loss of the organelle itself (Zhu, 2004). It also lost the whole mitochondrial genome, even though several mitochondrial genes encoded by the nucleus can be identified there (Abrahamsen et al., 2004; Xu et al., 2004). Thus, *C. parvum* does not posses many well-studied potential drug targets (e.g. HXGPRT, ODC, mannitol cycle, shikimate pathway, electron transport chain and apicoplast). However, the parasite does encode several other potential drug targets, some of which are unique, gained by horizontal transfer from other organisms such as plants or bacteria. Additionally it possesses some aspects that have been likely gained through endosymbiosis and contains proteins highly divergent or absent in humans and animals (Huang et al., 2004).

Our general understanding of the *C. parvum* metabolic machinery has significantly increased by now but our knowledge of many specific pathways and enzymes in *Cryptosporidium* is still limited. A better understanding of important metabolic pathways and enzymes in this organism would aid in the experimentation of new drugs and developing new strategies to treat this infection in humans and animals.

## AIMS OF THE DISSERTATION

This dissertation builds on a long term research goal of our laboratory. The focus of our laboratory is to characterize the molecular and biochemical features of enzymes involved in the core metabolism of *Cryptosporidium parvum* in an effort to characterize a new drug targets for novel therapeutics development.

The specific objectives of the research described in this dissertation are following:

- To specify the subcellular localization of one of the core metabolic enzymes, pyruvate:$NADP^+$ oxidoreductase, which is responsible for converting pyruvate to acetyl-CoA.

- To characterize a *Narf*-like gene in *Cryptosporidium* that resembles [Fe]-hydrogenases in other anaerobic protists.

- To characterize S-adenosylhomocysteine hydrolase from *C. parvum*. This is an important enzyme regulating the S-adenosylhomocysteine metabolic pathway considered important in the target-based drug design of antiviral and antiparasitic drugs.

- To validate adenosine analogues capable inhibiting both the recombinant enzyme S-adenosylhomocysteine hydrolase and *in vitro* growth of *C. parvum.*

## METHODS

The materials and methods were described in details in the published articles entitled: "Localization of Pyruvate:NADP+ Oxidoreductase in Sporozoites of *Cryptosporidium parvum*" (Ctrnacta *et al.*, 2006); "A *Narf*-like gene from *Cryptosporidium parvum* resembles homologues observed in aerobic protists and higher eukaryotes" (Stejskal *et al.*, 2003); "Characterization of S-adenosylhomocysteine hydrolase from *Cryptosporidium parvum*" (Ctrnacta *et al.*, 2007). The methods that have not been published yet have been described in details in the dissertation.

**Organisms**

Following organisms were used in the course of described studies: *Euglena gracilis* strain B without a plastid; *Cryptosporidium parvum*, Iowa strain: obtained oocysts were Clorox-treated and purified and excysted, sporozoites were used immediately for DNA and RNA isolation, microscopy or for infection of HCT-8 cells; human colorectal cell line HCT-8 that were used for parasite infection; *Escherichia coli* TB1 were used as a background organism for MBP-SAHH expression.

**Nucleic acid methods**

BLAST search, library screening, sequencing were performed for mentioned genes. The recovered DNA sequences of *CpNARF* and *CpSAHH* were translated to amino acids. Multiple protein sequence alignments of both CpNARF and CpSAHH with their homologue proteins from different taxa were created and related proteins compared. The conservative or active residues were identified. CpNARF protein including the H-cluster domains and one neighboring iron-sulfur ([4Fe-4S]) cluster were used to calculate trees in three different phylogenetic programs.

Total genomic DNA (gDNA) and RNA were isolated from *C. parvum* sporozoites and from mock-infected HCT-8 cells, as well as from cultures infected with *C. parvum*. For isolation, DNA Mini Kit (Qiagen) and RNeasy Mini Kit (Qiagen) were used according to manufacture's instructions. The isolated gDNA of *CpNARF* and *CpSAHH* was used for Southern blot assays and RT-PCR to analyze gene expressions.

The measurements of SAHH inactivation by adenosine analogues, in parasites that infected human HCT-8 cells, were based on quantitative real-time reverse transcription-PCR (qRT-PCR). A SYBR green-based real-time RT-PCR method was employed using the QuantiTect SYBR Green RT-PCR kit (Qiagen). Appropriate amounts of reagents and primers were first incubated at 48°C for 30 min to synthesize cDNA, heated at 95°C for 15 min to inactivate the reverse transcriptase, and then subjected to 40 thermal cycles (95°C for 20 s, 50°C for 30 s, and 72°C for 30 s) of PCR amplification with an iCycler iQ real-time PCR detection system (Bio-Rad). Analysis of Cp18S rRNA and Hs18S rRNA levels and the eventual calculations of parasite inhibition were calculated (Cai et al. 2005).

**Protein methods**

The full-length *CpSAHH* sequence was PCR amplified from *C. parvum* gDNA using sense F1 and antisense R1 primers. Gel-extracted amplicons of *CpSAHH* were ligated into the polylinker cloning site of the pMAL-c2x vector (New England Biolabs) cleaved with *Bam*HI and *Pst*I. The *pMAL-SAHH* construct was transformed to *E. coli* TB1 cells. Plasmids isolated from transformed cells were subjected to restriction analyses and subsequently sequenced to confirm their identities before

expression. Expression of the MBP-CpSAHH was induced with 0.3 mM IPTG for 15 h at 30°C. The MBP-CpSAHH fusion protein was affinity purified using amylose resin-based chromatography according to the manufacturer's instructions (New England Biolabs). Protein concentration was estimated using the Bradford assay with bovine serum albumin as a standard. The expression of MBP-CpSAHH was confirmed by SDS-PAGE and western blotting. MBP-CpSAHH was cleaved with Factor Xa and CpSAHH was further applied to a CHT 5-I hydroxyapatite column and purified.

The size of the native recombinant SAHH enzyme was analyzed under native conditions by size exclusion column chromatography (Superdex 200 10/300 GL column).

The enzymatic activities of both the MBP-CpSAHH fusion protein and cleaved CpSAHH were spectrophotometrically assayed in the hydrolytic direction (Yuan *et al.,* 1996; Lozada-Ramirez *et al*., 2006) using S-adenosylhomocysteine as a substrate. Inhibition assays were carried out by the preincubation of 5 µl of 500 µg/ml CpSAHH with different concentrations of Ara-A, S-DHPA and D-eritadenine for 15 min at 37°C. After incubation SAHH activity was assayed by adding 50 µM S-adenosylhomocysteine at a final volume of 1 ml to start the reaction. The same experiment was repeated four times with different inhibitor concentrations: 0, 10, 100, 500 nM and 1 µM.

Western blot assay was used to reveal the expression of the complete PNO protein in *C. parvum* and *E. gracilis.* Both primary polyclonal antibodies against PFO and CPR domains (prepared using synthetic oligopeptides by ProSci Inc. Poway, CA, USA) were used. As a control of antibody specifity, a peptide competition assay was employed. The oligopeptide used for producing antibody was preincubated with the appropriate antibody. Specificity was evaluated and confirmed by the lack of a signal on the western blot due to antibody interaction with the oligopeptide leaving no antibody to bind on the nitrocellulose membrane. The negative control included the preincubation of heterologous oligopeptides with the antibody in which case the signal on western blot should was detected.

**Microscopy: localization**

The polyclonal primary antibodies against both the PFO and CPR domains of CpPNO from rabbit and goat were used. As a control, a primary antibody against cytosolic fatty acid phosphopantetheinyl transferase (CpPPT) was used. CpPPT was a gift from Dr. Guan Zhu (Texas A&M University, College Station, TX, USA). Another control antibody against *C. parvum*-specific mitochondrial heat shock protein CpCpn60 was provided by Dr. Jan S. Keithly (Wadsworth Center, Albany, NY, USA).

Both confocal microscopy and transmission electron microscopy was used to detect the localization of CpPNO in *C. parvum* sporozoites.

## MAIN RESULTS

### Pyruvate:NADP$^+$ oxidoreductase

In many microaerophilic protists, oxygen-sensitive pyruvate:ferredoxin oxidoreductase (PFO), a key enzyme in core metabolism, is compartmentalized either within the cytosol (*Giardia, Entamoeba*) or within a double membrane-bounded hydrogenosome (*Trichomonas, Nyctotherus*) (Embley *et al.*, 2003). In *C. parvum*, PFO is the N-terminal domain of a unique fusion with NADPH-cytochrome P450 reductase (CpCPR) forming pyruvate:NADP$^+$ ferredoxin oxidoreductase (CpPNO), an enzyme rarely found in eukaryotes. Interestingly, CpPNO shares significant sequence homology and domain organization with *Euglena gracilis* PNO (EgPNO) (Inui *et al.*, 1984; Inui *et al.*, 1987). However, their function and localization is not identical.

Here was found that there was neither CpPNO activity detected in *C. parvum* sporozoites, nor EgPNO-like activity in spite of detectable EgPNO activity in the *E. gracilis* positive control (Hrdy, Stejskal, Ctrnacta, unpublished results). The forward reaction of this enzyme was assayed using pyruvate as an electron donor, and methyl viologen, NADP$^+$, NAD$^+$ or FAD as electron acceptors (Inui *et al.*, 1987). The reverse reaction in *C. parvum* was assayed using methods well-established for *E. gracilis*, clostridia and photosynthetic bacteria (Inui *et al.*, 1987). Positive controls included both mitochondrial EgPNO and the hydrogenosomal *Trichomonas vaginalis* PFO. It is difficult to learn why CpPNO, but not its activity, is present in sporozoites without an efficient *in vitro Cryptosporidium* cultivation system. The quantity of CpPNO may be below the detection limits of our enzymatic assays in sporozoites, or the functional protein may only be expressed by intracellular stages multiplying in the intestinal epithelium under microaerophilic conditions, similar to that for the PFO of diplomonads, entamoebids and trichomonads.

*C. parvum* specific polyclonal antibodies against CpPFO, CpCPR, the cytosolic fatty acid phosphopantetheinyl transferase (CpPPT) and the mitochondrial heat shock protein (CpCpn60) were used for confocal immunofluorescence microscopy co-localization and TEM experiments. Using confocal immunofluorescence and TEM, it has been shown that CpPNO is localized within the cytosol rather than within the relict mitochondrion. Interestingly, both immunofluorescence and TEM experiments showed that CpPNO is also localized in the crystalloid body (CB) - an organelle whose function is currently unknown. The size of the CB is significant as it is at least equal in volume to the nucleus. The 3-D reconstruction of this organelle indicates that the closely packed vesicles observed by TEM might be a series of interconnected channels. This organelle is always closely positioned to the relict mitochondrion, outer nuclear membrane and rough-endoplasmic reticulum (Keithly *et al.*, 2005). Even though there were no clear observations of limiting membrane made, we showed that the CB takes up MitoTracker Green FM dye, suggesting there is some kind of membrane, possibly with an organellar membrane potential.

**[Fe]-hydrogenase or NARF-like protein?**

The traces of [Fe]-hydrogenase genes can be found everywhere, even in our own genome and in all higher eukaryotes analyzed so far. However, these genes code proteins that lack detectable hydrogenase activity and probably cannot produce molecular hydrogen, has been called NARF-like proteins. Their *Narf*-like genes show extensive similarity to the genes of [Fe]-hydrogenases that, on contrary, are metabolically active proteins containing an N-terminal active site with [Fe-S] clusters, usually located in hydrogenosomes (Nicolet *et al.*, 2000).

In *C. parvum*, a [Fe]-hydrogenase-like fragment was identified. The entire ORF was cloned into a pCR2.1 TOPO vector and sequenced. The nucleotide sequence of *CpNARF* gene was deposited to GenBank. The Southern blot analysis revealed that *CpNARF* is a single copy gene. Its expression was confirmed in both extracellular and intracellular stages of the parasite by RT-PCR.

In *C. parvum*, i) the residues of H-cluster (composed of [4Fe-4S] and [2Fe] clusters) and FS4A domain (an adjacent medial Fe-S cluster) have been identified; ii) there was no electron accepting domain and surprisingly neither organellar targeting signal or nuclear localization sequences were detected; iii) a putative proton donating C conserved in hydrogen producing hydrogenases was not found in *CpNARF*; iv) alike most of NARF like-proteins, CpNARF was found to contain a conserved W residue at the C-terminus. Furthermore, [Fe]-hydrogenases enzymatic activity could not be detected in *C. parvum* sporozoite extract using standard assays (Hrdy and Stejskal, unpublished data).

Multiple amino acid sequence alignment of [Fe]-hydrogenases with NARF-like proteins revealed a close relationship of *C. parvum* protein with NARF-like proteins from aerobic protists and higher eukaryotes instead of [Fe]-hydrogenases of microerophilic protists and anaerobic bacteria. This was confirmed by phylogenetic analysis of 37 taxa as it revealed that the gene from *C. parvum* clusters with the *Narf*-like genes from yeast, animals and higher plants and not with [Fe]-hydrogenases of anaerobic protist (*E. histolytica, G. intestinalis, T. vaginalis, Nyctotherus ovalis*). Thus, *C. parvum* gene has been classified as a *Narf*-like gene and was named *CpNARF*.

**S-adenosylhomocysteine hydrolase**

In *C. parvum*, S-adenosylhomocysteine hydrolase (SAHH) is the only known enzyme to catalyze the breakdown of S-adenosylhomocysteine (AdoHcy) to homocysteine and adenosine. AdoHcy is the product of all adenosylmethionine (AdoMet)-dependent biological transmethylations. Its decarboxylated form is a donor of an aminopropyl group for polyamine synthesis. As a product inhibitor, elevated levels of AdoHcy suppress AdoMet-dependent transmethylations. Thus, SAHH is a regulator of biological transmethylation in general (Turner *et al.,* 2000).

Using Southern blot analysis, it was revealed that CpSAHH is present in the genome as a

single copy gene which was later confirmed by whole genome sequencing (Abrahamsen *et al.*, 2004). The CpSAHH expression profile was determined with RT-PCR analysis, which showed that the *CpSAHH* gene was expressed both in sporozoites and in the intracellular stages multiplying in HCT-8 cells. Multiple sequence analysis of CpSAHH and other selected prokaryotic and eukaryotic SAHH homologues indicated that CpSAHH contains most of the amino acid residues and motifs necessary for enzymatic activities. The complete *CpSAHH* gene was cloned into the pMAL-c2X vector and was expressed in *E. coli* TB1 cells as an MBP-fusion protein. After purification, the cleaved denaturated protein was subjected to SDS-PAGE to establish its subunit molecular mass and the recombinant native protein was subjected to the size-exclusion chromatography on a Superdex 200 column. The apparent molecular mass of the native protein suggested that C*p*SAHH is a tetramer which is in agreement with SAHH proteins in variety of organisms (Fujioka and Takata, 1981; Porcelli *et al.*, 1993; Tanaka *et al.*, 2004).

As was predicted from amino acid sequence comparisons, CpSAHH retained its enzymatic activity. Its activity was tested spectrophotometrically in the hydrolytic direction. The enzyme kinetic profiles ($K_m$, $K_{cat}$, $V_{max}$) of both CpSAHH and MBP- CpSAHH were determined. Results showed that the kinetic profiles of CpSAHH and MBP-CpSAHH differed as the MBP tag seemed to slow down the enzymatic reaction. However, in comparison to known kinetic profiles from other organisms, CpSAHH falls within their range. The catalytic turnover of CpSAHH was determined to be $K_{cat} = 0.69$ $s^{-1}$ which falls in the range of known values $K_{cat} = 0.13 – 3.8$ $s^{-1}$ (Fujioka and Takata, 1981; Yuan *et al.*, 1996; Porcelli *et al.*, 2000).

A pilot experiment with recombinant protein revealed that three compounds were able to inhibit CpSAHH at low concentrations. Ara-A has been approved and primarily used as an antiviral agent that displays a wide antiviral spectrum. In agreement with inhibition of recombinant SAHH from *T. vaginalis* (Minotto *et al.*, 1998), Ara-A at 500 nM inhibited CpSAHH by 17.5%. The other two inhibitors are adenosine analogues with acyclic sugar moieties and both of them are known potent antiviral agents (Holy *et al.*, 1985). Neither of them has been tested against SAHH from parasitic protozoa. (S)-DHPA displayed similar inhibition as Ara-A. At a concentration of 500 nM the enzymatic activity of CpSAHH remained at the 86% level, so (S)-DHPA inhibited CpSAHH by 14%. However, D-eritadenine proved to be very effective inhibitor of CpSAHH. At 500 nM, it inhibited CpSAHH activity by 95.3%, the remaining enzymatic activity was 4,7%. D-eritadenine seemed to be a very promising drug especially in the light of the previous success as an effective antiviral agent.

All mentioned inhibitors were further tested in the microenvironment of the host cell. We employed qRT-PCR assay to evaluate the efficiency of used aliphatic inhibitors, which were previously shown to have also antiviral activity (Holy et al., 1985), on the growth of *C. parvum in vitro*. The relative expression was determined by detection of both host cell 18S rRNA ($C_{T[H18S]}$) as an internal control and parasite 18S rRNA ($C_{T[P18S]}$). The level of parasite 18S rRNA was subsequently calculated as a ratio of $C_T$ values ($\Delta C_T = C_{T[P18S]} - C_{T[H18S]}$). The data was further processed and

statistically analyzed (Cai et al., 2005). All used inhibitors displayed a dose-dependent inhibition of the intracellular stages of *C. parvum* cultured with HCT-8 cells. The $IC_{50}$ was determined by nonlinear curve regression. The $IC_{50}$ values for (S)-DHPA, (R)-DHPA and D-eritadenine were determined to be 4.3 μM, 11.6 μM and 44.8 μM, respectively. No significant cytotoxicity to HCT-8 cells was observed at used concentrations by the MTT assay (a standard colorimetric assay for measuring cellular growth and cytotoxicity of potential medicinal agents). The drug efficiencies were compared with two golden standards used in drug testing against *C. parvum*: paromomycin, a compound that inhibits protein synthesis by binding to 18S rRNA (Marshall and Flanigan, 1992) and nitazoxanide (NTZ), a drug, which is approved for the treatment of infectious diarrhea caused by *C. parvum* and *G. intestinalis* in patients 1 year of age and older in USA (Bailey and Erramouspe, 2004). After comparison, the data showed that inhibition of the growth of *C. parvum in vitro* with (S)-DHPA, (R)-DHPA and D-eritadenine, respectively, was more potent than when using paromomycin ($IC_{50}$ = 137 μM) (Cai et al., 2005). The potential to inhibit intracellular stages of *C. parvum* was also elucidated with NTZ from other study ($IC_{50}$ = 0.98 μM) (Cai et al., 2005). The inhibition of CpSAHH using (S)-DHPA was efficient enough even though it did not reach the level of NTZ. Nevertheless, it can be claimed that (S)-DHPA and (R)-DHPA may be explored as potential drug targets for the control of *C. parvum* infection. Interestingly, the most potent inhibitor of the recombinant protein, EritA was the least potent inhibitor of the CpSAHH in *in vitro* experiment. This results is in agreement with previous studies (Holy et al., 1985) describing that particular inhibitors can be very potent against isolated and recombinant proteins but to have only minimal effect on SAHH catabolism in intact cells or vice versa.

**DISSCUSSION**

The most significant divergence of amitochondrial organisms from those with mitochondria is in the mechanism of oxidative decarboxylation of pyruvate to acetyl-CoA, a central step of core energy metabolism. All organisms with an aerobic mitochondria utilize the pyruvate dehydrogenase complex (PDH), which consists of the stepwise transfer of electrons via an acetyl group to CoA, then from dihydrolipoamide to FAD, and finally to $NAD^+$. The other system occurs in anaerobic prokaryotes and microaerophilic eukaryotes including *Trichomonas vaginalis*, *Entamoeba histolytica*, and *Giardia intestinalis*. The enzyme responsible for oxidative decarboxylation in these organisms is the iron-sulphur protein, **pyruvate feredoxin oxidoreductase** (PFO), which converts pyruvate to acetyl-CoA with the transfer of a pair of electrons to ferredoxin (Muller, 2003). In contrast to all other anaerobic protists and prokaryotes, *Cryptosporidium* utilizes a unique enzyme for oxidative decarboxylation, **pyruvate-NADP oxidoreductase** (PNO). Only other described PNO had been characterized well in *Euglena gracilis* (Inui *et al.,* 1987) In *E. gracilis* PNO catalyzes both a reversible pyruvate oxidation by $NADP^+$ and a pyruvate $CO_2$ exchange, similar to the reactions catalyzed by

PFO. Unlike PFO, the PNO of *Euglena* does not react with ferredoxin (Rotte *et al.*, 2001). However, CpPNO exact enzymatic function does not to be identical and has not been characterized yet. In our laboratory, no CpPNO activity in *C. parvum* sporozoites was detected, in spite of detectable EgPNO activity in the *E. gracilis* positive control (Hrdy, Stejskal, Ctrnacta, unpublished results). Because *Cryptosporidium* relies solely on glycolysis as an energy source, CpPNO might play a crucial role in the carbohydrate metabolism. This role and the absence of PNO in mammalian cells suggest this enzyme is a good potential drug target.

What concerns the localization of PNO in *C. parvum*, it is well-established that the PFO of certain parasites can be either cytosolic (e.g. in *G. intestinalis*, *Entamoeba histolytica*) or organellar (e.g. in *T. vaginalis, Nyctoterus ovalis*) within double membrane bounded hydrogenosomes (Boxma et al., 2005; Muller, 2003). In this dissertation, it has been shown that CpPNO is localized within the cytosol rather than within the relict mitochondrion and surprisingly, CpPNO was also concentrated in crystalloid bodies. The function of the CB in *C. parvum* might be consistent with the function of refractile bodies in *Eimeria tenella* that are suggested to contain numerous proteins and lipids that might play a part during invasion process into the host cell (deVenevelles *et al.*, 2006). So hypothetically, CpPNO localization within the CB may indicate the presence of additional ATP generation to power the invasion of the parasite to the host cell (Ctrnacta *et al*., 2006).

In *Trichomonas vaginalis*, pyruvate is oxidized by PFO and generated electrons are transferred to ferredoxin which, under anaerobic conditions, is reoxidized by **[Fe]-hydrogenase** that produces the molecular hydrogen (Muller, 1993). Because [Fe]-hydrogenase-like fragment had been identified in the *C. parvum* genome, we decided to determine whether *C. parvum* may contain both PFO and [Fe]-hydrogenase genes. Phylogenetic analyses revealed that gene identified in *C. parvum*, translated to the putative protein belongs to the group of **NARF-like proteins** from aerobic protists and higher eukaryotes, which are thought to have had an ancestor in common with [Fe]-hydrogenases. [Fe]-hydrogenases appear to have diverged in both structure and function as core metabolism changed in aerobic and anaerobic eukaryotic lineages over evolutionary time. Even though we do not know its function, according to obtained data, *C. parvum* gene has been classified as a *Narf-like* gene and was named *CpNARF* (Stejskal *et al*., 2003). *Narf-like* genes are shorter then [Fe]-hydrogenase genes and some of them contain nuclear localization sequences. NARF-like proteins localization together with their absence of hydrogen production suggests that these proteins are probably not involved in energy metabolism (Horner et al., 2000). It was confirmed recently by showing that yeast Narf-like protein Nar1 was involved in the cytoplasmic and nuclear [Fe-S] metabolism (Balk et al., 2004).

Amino acids containing sulphur: methionine and cysteine, are a part of virtually every protein. Despite sulphur amino acids general utility, sulfur itself is toxic and its handling needs to be carefully regulated. Its regulation includes transsulfuration, sulfur assimilatory de novo cysteine biosynthesis,

methionine cycle and degradation which involve at least two dozen intermediates and products. If these sulphur containing amino acids and some of their intermediates are not completely metabolized, their accumulation can have a very dangerous and even fatal impact to the overall metabolism. Although these two amino acids are indispensable for all living organisms, there are remarkable differences in their biosynthesis and catabolism between parasitic protozoa and their mammalian hosts (Nozaki et al., 2005). Our understanding of these differences is crucial as it can help us to identify and exploit unique targets to develop novel chemotherapeutic and prophylactic agents. One of the important sulphur-containing intermediates is S-adenosylhomocysteine. This compound is catabolized by the enzyme **S-adenosylhomocysteine hydrolase** (SAHH) into adenosine and L-homocysteine (De La Haba and Cantoni, 1959). If SAHH is inhibited, S-adenosylhomocysteine accumulates in the cytoplasm where it acts as a potent feedback inhibitor and blocks methylation reactions by inhibiting S-adenosylmethionine-dependent methyltransferases, thus influencing the overall methylation capacity of the cell (Turner *et al*., 2000). SAHH has been isolated from many organisms including humans (Hershfield *et al*., 1985), rats (Fujioka and Takata, 1981), plants (Sebestova *et al.,* 1984), yeast (Knudsen and Yall, 1972), protozoa: *Trichomonas vaginalis* (Bagnara et al., 1996), *Plasmodium falciparum* (Creedon *et al.,* 1994), prokaryotes (Porcelli *et al*., 2005) and characterized. Even though SAHH has been studied extensively as a potential drug target for a long time, it has not yet been proposed to be used as a target protein against *Cryptosporidium*. In the dissertation, CpSAHH was characterized for the first time (Ctrnacta *et al*., 2007). It was shown that this protein is also suitable as a drug target against this parasite. Several aliphatic inhibitors with antiviral activities (Holy *et al*., 1985) were tested on the recombinant CpSAHH enzyme as well as in *in vitro* system where they were able to inhibit the growth of *C. parvum* in host human HCT-8 cells and simultaneously not to be toxic to the human cells. Currently in our laboratory, the search for other potential inhibitors of SAHH is going on and more inhibitors are being tested to ultimately find the most effective and safe inhibitor of growth *C. parvum* in human cells. We are not alone in this search, there are many laboratories searching for other suitable drug targets in *C. parvum* as well as for the functional, well defined inhibitors.

## SUMMARY

Via the fulfillment of given research objectives in this dissertation the results presented bring a characterization of three potential drug targets from *C. parvum*: pyruvate:$NADP^+$oxidoreductase, S-adenosylhomocysteine hydrolase and a *CpNARF*:

**Pyruvate:NADP+ oxidoreductase**

- In contrast to PNO in *Euglena gracilis*, CpPNO is a cytosolic, not mitochondrial protein, which was confirmed by both confocal immunofluorescence and immunogold labeling for TEM. This new data is in agreement with the suggestion that the relict mitochondrion of *C. parvum* serves primarily as an organelle for the import and maturation of [Fe-S] clusters and that it plays no major role in core energy metabolism.

- Both confocal images and immunogold labeling showed that CpPNO is localized also within the crystalloid body, as well as within the cytosol. These data, together with the close juxtaposition of the CB and the relict mitochondrion, as well as their distinctive internal structure, indicate that there may be some interesting evolutionary implications for why the CB remains a unique subcompartment within *C. parvum*. Because CpPNO is compartmentalized in a novel way, it opens the possibility that this apicomplexan may display yet another unique type of core energy metabolism in microaerophilic protists that might lead to new strategies for drug development against human cryptosporidiosis.

- The uptake and localization of fluorescent dyes (Mitotracker Green FM, Rhodamine B and Rhodamine 123) into both the CB and the relict mitochondrion was shown, which might indicate an organellar membrane potential in spite of neither TEM nor tomographic reconstruction could resolve whether the CB is membrane bounded.

***CpNARF* gene**

- In *C. parvum*, an [Fe]-hydrogenase-like gene had been identified and characterized. This gene contains a single highly conserved N-terminal iron-sulfur cluster ([4Fe-4S]) binding site, as well as most of the H-cluster conserved residues and lacks the obvious N-terminal organelle-targeting signal, present in most metabolically active [Fe]-hydrogenases.

- RT-PCR analysis revealed that *CpNARF* gene is expressed by the intracellular stages of *C. parvum* and it encodes putative protein of 560 amino acids, which was named CpNARF.

- Phylogenetic analysis revealed that *CpNARF* gene from *C. parvum* clustered with *Narf*-like genes from yeast, animals and higher plants. *CpNARF* gene did not cluster with [Fe]-hydrogenases of anaerobic protists (*E. histolytica, G. intestinalis, S. barkhanus, T. vaginalis, Nyctotherus ovalis*).

- Although the function of this gene is unknown, both phylogenetic analyses and sequence data suggest that CpNARF belongs to the group of NARF-like proteins from aerobic protists and higher eukaryotes, which are thought to have had an ancestor in common with [Fe]-hydrogenases.

**S-adenosylhomocysteine hydrolase**

- CpSAHH has been cloned and analyzed. The protein differs substantially from mammalian SAHH homologues and other nonapicomplexan protists as it contains a 49 amino acid plant-like insertion.

- CpSAHH is expressed both in intracellular stages (in *C. parvum*-infected HCT-8 cells) and in sporozoites.

- CpSAHH was expressed as a fusion protein MBP-CpSAHH, cleaved and purified. Enzymatic activities of both MBP-CpSAHH and CpSAHH ($K_m$, $V_{max}$, $K_{cat}$) were determined using the Michaelis-Menten plot. When compared to known kinetic profiles of SAHH from other organisms, CpSAHH falls within their range.

- Tertiary structure of the recombinant CpSAHH is a tetramer like most of the studied SAHH proteins.

- The enzymatic activity of CpSAHH was inhibited by D-eritadenine, (S)-DHPA and Ara-A. The most efficient inhibitor of the recombinant CpSAHH was D-eritadenine; 500 nM D-eritadenine inhibited CpSAHH activity by 95.3%

- Inhibitors (S)-DHPA, (R)-DHPA and D-eritadenine were observed to inhibit *C. parvum* growth *in vitro*, in infected human HCT-8 cells. The $IC_{50}$ values for S-DHPA, R-DHPA and D-eritadenine were 4.3 μM, 11.6 μM and 44.8 μM, respectively. Based on results presented in this dissertation both (S)-DHPA and (R)-DHPA were proven to be good candidates for next step in drug development against cryptosporidiosis.

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