EXPLORING THE GLYCOLYTIC ENZYMES, GLUCOSE-6-PHOSPHATE ISOMERASE (CpGPI) AND HEXOKINASE (CpHK) AS POTENTIAL DRUG TARGETS IN *CRYPTOSPORIDIUM PARVUM*

A Dissertation

by

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ABSTRACT

Cryptosporidium parvum is a water-borne and food-borne apicomplexan pathogen. It is one of the top four diarrheal-causing pathogens in children under the age of five in developing countries, and an opportunistic pathogen in immunocompromised individuals. The preventative measures are not fully effective with, nitazoxanide (NTZ), the only FDA-approved drug for use in immunocompetent individuals. Unlike other apicomplexans, *C. parvum* lacks Kreb's cycle and cytochrome-based respiration, thus relying mainly on glycolysis to produce ATP. In this study, we characterized the primary biochemical features of the *C. parvum* glucose-6-phosphate isomerase (CpGPI) and determined its Michaelis constant towards fructose-6-phosphate (K_m = 0.309 mM, V_{max} = 31.72 nmol/μg/min). We also discovered that ebselen, an organoselenium drug, was an inhibitor of CpGPI by high-throughput screening of 1,200 known drugs. Ebselen acted on CpGPI as an allosteric noncompetitive inhibitor (IC_{50} = 8.33 μM), while complete inhibition of CpGPI activity was not achieved. Although ebselen is useful in studying the inhibition of CpGPI enzyme activity, further proof is needed to chemically and/or genetically validate CpGPI as a drug target. We also identified four drugs as CpHK inhibitors with micromolar level of anti-cryptospordial activities at concentrations nontoxic to the host cells (i.e., hexachlorphene, thimerosal, alexidine dihydrochloride and ebselen with EC_{50} = 0.53, 1.77, 8.1 and 165 μM, respectively). The anti-CpHK activity of the four existing drugs provided us new reagents for studying the enzyme properties of the parasite hexokinase. We have previously observed that 2-deoxy-D-glucose (2DG) could inhibit both the enzyme activity of *C. parvum* hexokinase (CpHK) and the parasite growth in vitro. However, the action and fate of 2DG in *C. parvum* was not fully investigated. In the present study, we showed that, although 2DG could be phosphorylated by CpHK to form 2DG-6-phosphate (2DG6P), the anti-cryptosporidial activity

of 2DG was mainly attributed to the action of 2DG on CpHK, rather than the action of 2DG or 2DG6P on the downstream enzyme CpGPI, nor 2DG6P on CpHK. These observations further supported the hypothesis that CpHK could serve as a drug target in the parasite.

DEDICATION

TO

MY PARENTS

For their endless love and care without whom none of my success will be possible

MY SISTERS

For always encouraging, helping me and setting example for me all over the years

MY HUSBAND

For always loving, supporting and believing me with a constant source of positive energy

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NOMENCLATURE

ACC	Acetyl-CoA carboxylase
AceCL	Acetic acid-CoA ligase
ACL	Acyl-CoA Ligase
ADE	Aldehyde deydrogenase
ADH1	Alcohol dehydrogenase 1
AIDS	Acquired Immune Deficiency Syndrome
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CDC	Centers for Disease Control and Prevention
CoA	Coenzyme A
COWP	*Cryptosporidium* Oocyst Wall Protein
CTP	Cytidine Triphosphate
DG	Deoxy-glucose
DG6P	Deoxy-glucose-6 phosphate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
Ebs	Ebselen
FBS	Fetal Bovine Serum
F6P	Fructose-6 phosphate

FDA	Food and Drug Administration
GDBE	Glycogen debranching enzyme
gDNA	Genomic Deoxyribonucleic Acid
GP	Glycogen phosphorylase
GTP	Guanosine Triphosphate
G6P	Glucose -6phosphate
G6PDH	Glucose-6- phosphate dehydrogenase
HAART	Highly-Active Anti-Retroviral Therapy
HCT-8	Human Colonic Tumor 8
HK	Hexokinase
HIV	Human Immunodeficiency Virus
Hs	Homo sapiens
ICZN	Code of Zoological Nomenclature
IFN-γ	Interferon Gamma
IL-12	Interleukin 12
IPTG	Isopropyl-1-Thio-β-D Galactopyranoside
LDH	Lactic acid dehydrogenase
MBP	Maltose Binding Protein
MDH	Malate dehydrogenase
MHC	Major Histocompatibility Complex
MTT	Methylthiazol Tetrazolium
NCBI	National Center for Biotechnology Information
NIH	National Institutes of Health

NTP	Nucleoside Triphosphates
NTZ	Nitazoxanide
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PEP	Phospho-enol pyruvate
PCR	Polymerase Chain Reaction
PDC	Pyruvate decarboxylase
PGI	Phosphoglucose isomerase
PGluM	Phosphoglucose mutase
PI	Post-Infection
PK	Pyruvate kinase
PMI	Phosphomannose isomerase
PNO	Pyruvate:$NADP^+$ oxidoreductase
PPi-PFK	Pyrophosphate-dependent phosphofructokinase.
PV	Parasitophorous Vacuole
PVM	Parasitophorous Vacuolar Membrane
qRT-PCR	Quantitative Reverse Transcriptase-Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction SCID
	Severe Combined Immune Deficient
SDS	Sodium Dodecyl Sulfate

TAMU	Texas A&M University
TCA	Tricarboxylic acid cycle
UTP	Uridine Triphosphate
WHO	World Health Organization

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1 INTRODUCTION AND LITERATURE REVIEW

1.1 The history of *Cryptosporidium* spp.

In 1970, *Cryptosporidium* was first recognized by Ernest Edward Tyzzer in gastric glands of laboratory mice with both its asexual and sexual phases of reproduction [1]. He thought the oocysts excreted were spores so he identified the parasite as a sporozoan and name it *Cryptosporidium muris*. He recognized all life cycle morphological features except being an intracellular rather than extracellular parasite. In 1910, he suggested autoinfection might occur in Japanese waltzing and English mice [2]. Then, he described *Cryptosporidium parvum* as another new species that develops smaller oocysts that those of *C.muris* and infecting the small intestine of mice [3]. In 1948, the first case associated with fatality was in turkeys by the new species, *Cryptosporidium meleagridis* [4]. Yet, interest was fair for this parasite. Later in 1970, diarrheal cases in cattle were reported for which more interested was given. First 2 cases of human cryptosporidiosis were reported, a 3-years-old child and a 39-years-old immunosuppressed patient. Both cases were in contact cattle and a dog while presenting watery diarrhea [5]. In 1979, another case was reported by 9 -years-old child and 52-years-old immunosuppressed patient [6]. Six out of the seven cases reported between 1976 and 1980 were immunosuppressed or immunocompromised [6]. Among the 58 cases reported from 1976 till 1984, 18 cases were immunocompetent [7]. Therefore, *Cryptosporidium* became a significant opportunistic human pathogen that is capable of infecting both immunocompromised and immnunocompetent patients. Cryptosporidial infections might result in severe and chronic diarrhea in immunocompromised patients, or mild to severe symptoms but usually self-limiting in immunocompetent individuals.

In the 1970s, more attention was driven to this pathogen owing to the water-borne

outbreaks of cryptosporidiosis worldwide. An outbreak was documented in Carrollton, Georgia, United States of America (USA), resulting in 13,000 cases due to the contamination of a filtered public water supply [8]. Another outbreak has affected 5,000 cases in Wiltshire and Oxfordshire, UK [9]. In 1993, the most influential waterborne outbreak in Milwaukee, Wisconsin, USA, caused a surge in interest to the public, government and environmental supporters. This outbreak not only resulting in 403,000 cases with watery diarrhea and 4,400 people were hospitalized but also associated with total costs around $96.2 million [10, 11].

Cryptosporidium is listed as a category B priority agent in the Biodefense program by the National Institutes of Health (NIH) and the Centers for Disease Control and Prevention (CDC) in the USA due to the moderate level of illness, ease of dissimination and level of survellience [12]. Accordingly, subsequent studies were initiated towards the development of new tools for pathogen detection and understanding its biology. Recently, our knowledge of *Cryptosporidium* parasites regarding taxonomy, epidemiology, transmission, pathogenesis and molecular biology has enormously expanded for the ultimate goal which of preventing and treatment of cryptosporidiosis. A highlight in the research era is the complete genome sequences of *C. parvum* (Iowa stain) and *C. hominis* (isolate TU502 isolate), which revealed the unique biological features in this opportunistic pathogen [13, 14]. Never the less, more cases and outbreaks are still reported with not fully effective preventative and effective procedures.

1.2 Taxonomy

The genus of *Cryptosporidium* are unicellular protozoans that belong to the Phylum Apicomplexa, which mostly have complicated life cycles. Apicomplexans possess an apical

complex which contains conoid, rhoptries, micronemes, apical rings and dense granules. These structures are important for cell invasion and locomotion. In spite of various debates, *Cryptosporidium* is placed under the Class Coccidea as an intestinal coccidian due to the similarity in parasite cycle and morphology with other coccidian species [15-17]. However, *Cryptosporidium* shows significant biological differences among other coccidians, (a) It is an intracellular but extracytoplasmic within the host cells [18]; (b) it has thick-walled and thin-walled oocysts, rather than only thick-walled oocysts ; (c) the small size of oocyst and lack of sporocyst [2]; (d) the lack of apicoplast and its genome [19]; and (e) the insensitivity to most anticoccidial drugs [20].

More recently, the molecular and phylogentic evidences suggest a closer relationship to gregarines. Recently, molecular phylogenic data shows *Cryptosporidium* is an early evolution branch at the base of the Apicomplexa, rather than as a sister to the other intestinal coccidia [16, 21, 22]. Moreover, it is also suggested that *Cryptosporidium* is more closely related to the to the gregarines than to the coccidia. Whether, *Cryptosporidium* has evoloved from or sister to the gregarines, it is not yet determined [23, 24]. However, *Cryptosporidium* lacks various metabolic pathways such as de novo synthesizing amino acids, pyrimidines, and purines which are present in the gregarines [13] [22].

Up to date, there are 25 valid *Cryptosporidium* species (Table 1.1) with more than 40 *Cryptosporidium* isolates referred as genotypes and requires more morphological, biological, and molecular data to determine whether they are valid new species [25]. Earlier, the species of *Cryptosporidium* are determined according to the host harboring the infective (oocyst) stage. *C. muris* in the mouse gastric glands and *C. parvum*, in the mouse intestine were the first 2 species discovered by Ernest Edward Tyzzer [1, 3]. Several *Cryptosporidium* species

were mistakenly named according to the host origin. *C. parvum* which infects wide range of host shows low infectivity rate in adult mouse. Therefore, the parasite discovered by Tyzzer in the laboratory mice and able to infect adult mouse can be the *Cryptosporidium* mouse genotype. It is the cross-transmission experiments in 1980s that validated *C. parvum* in which it can be transmitted between mice and cattle host species [18, 26]. Additionally, several newly named species were merged to *C. parvum* [27] [28, 29]. Many coccidian species were identified according to their distinctive oocyst morphology which can not be applied to *Cryprosporidium* because it lacks these unique features and can not be differentiated from each other. Also, morphological features of life cycle stages can aid in speciation but it is not feasible to examine each host with the unique features. More recently, genetic markers have opened new avenues for defining new *Cryptosporidium* spp. as these derived from genes encoding 18S ribosomal RNA (rRNA), the hyper-variable 60-kDa glycoprotein (gp60), and the oocyst wall protein (COWP)[25, 30]**.** Since one criterion was not enough to define *Cryptosporidium* spp, the combination of several criteria were needed in which the following needs to be fulfilled: (I) oocyst morphology; (II) genetic characterization; (III) demonstration of natural and, whenever feasible, experimentally defined host specificity; (IV) compliance with International Code of Zoological Nomenclature (ICZN) [25, 31, 32].

Table 1 The twenty-five valid named Cryptosporidium species and their host types [33]

Species name	Author(s)	Type hosts	Major host
C. muris	Tyzzer (1907, 1910)	Tame mice	Rodents
C. wrairi	Vetterling *et al*. (1971)	*Cavia porcellus* (Guinea pig)	Guinea pigs
C. felis	Iseki (1979)	*Felis catis* (Cat)	
C. serpentis	Levine (1980)	*Elaphe guttata, E. subocularis, Sanzinia madagascarensus* (Snakes)	Snakes and lizards
C. meleagridis	Slavin (1955)	*Meleagris gallopavo* (Turkey)	Birds and humans
C. parvum	Re: Upton and Current (1985) Tyzzer (1912)[a]	*Bos taurus* (Cattle)	Ruminants
C. baileyi	Current *et al*. (1986)	*Gallus gallus* (Chicken)	Birds
C. varanii	Pavlásek *et al*. (1995)	*Varanus prasinus* (Emerald Monitor)	Lizards
C. andersoni	Lindsay *et al*. (2000)	*Bos taurus* (Cattle)	Cattle
C. canis	Fayer *et al*. (2001)	*Canis familiaris* (Dog)	Dogs
C. molnari	Alvarez-Pellitero and Sitja-Bobadilla (2002)	*Sparus aurata* and *Dicentrarchus labrax* (Fish)	Fish
C. hominis	Morgan-Ryan *et al*. (2002)	*Homo sapiens* (Human)	Humans
C. galli	Re: Ryan *et al*. (2003*c*) Pavlásek (1999)[a]	Spermestidae, Frangillidae, *Gallus gallus, Tetrao urogallus, Pinicola enucleator* (Birds)	Birds
C. suis	Ryan *et al*. (2004)	*Sus scrofa* (Pig)	Pigs
C. bovis	Fayer *et al*. (2005)	*Bos taurus* (Cattle)	Cattle
C. fayeri	Ryan *et al*. (2008)	*Macropus rufus* (Kangaroo)	Marsupials
C. fragile	Jirku *et al*. (2008)	*Duttaphrynus melanostictus* (Toad)	Toads
C. macropodum	Power and Ryan (2008)	*Macropus giganteus* (Kangaroo)	Marsupial
C. ryanae	Fayer *et al*. (2008)	*Bos taurus* (Cattle)	Cattle
C. xiaoi	Fayer *et al*. (2010)	*Ovis aries* (Sheep)	Sheep and goats
C. ubiquitum	Fayer *et al*. (2010)	*Bos taurus* (Cattle)	Ruminants, rodents, primates
C. cuniculus	Re: Robinson *et al*. (2010) Inman and Takeuchi (1979)[a]	*Oryctolagus cuniculus* (European rabbit)	Rabbits
C. tyzzeri	Re: Ren *et al*. (2012) Tyzzer (1912) (*C. parvum*)[a]	*Mus musculus* (Mouse)	Rodents
C. viatorum	Elwin *et al*. (2012*b*)	*Homo sapiens* (Human)	Humans
C. scrofarum	Kváč *et al*. (2013*b*)	*Sus scrofa* (Pig)	Pigs
C. erinacei	Kváč *et al*. (2014*b*)	European hedgehog (*Erinaceus europaeus*)	Hedgehogs and horses

1.3 Morphology of life cycle stages

The life cycle of *Cryptosporidium* spp. is complicated. It contains 6 developmental stages with different morphological features as shown by light and electron microscopy. The main site of infection of *C. parvum* (Human and other animals) and *C. hominis* (exclusive in human) is the small intestine. Sites may vary according to the host. For example, the ileum above the cecal junction is the favorable site for *C. parvum* in mice and calves. Infection can also occur in extra intestinal sites as respiratory system, gall bladder and liver in animals and immunocompromised patients [34].

1.3.1 Oocysts

The oocysts of *Cryptosporidium* spp. are round to oval in shape, with sizes ranging from 3.7 to 8.4 μm in length and 3.2 to 6.3 μm in width. *Cryptosporidium* oocyst wall is a trilaminar structure. The outer layer is an irregular electron-dense layer (8.5 ± 0.6 nm) while the middle layer is translucent (4.0 ± 0.2 nm). The inner layer is made of two electron-dense layers (13.0 ± 0.5 nm and 18.6 ± 1.6 nm, respectively). The thin walled oocysts lack the thin inner wall. [35, 36]. The wall has a suture structure through which sporozoites are released during excystation [37]. Each mature oocyst contains four sporozoites, and one membrane-enclosed residual body with large lipid body inside. The residual body has a large lipid body, protein inclusions and amylopectin granules [38]. The tough oocyst wall is mainly the reason of being resistant to common disinfection techniques for water treatment as chlorination [39].

1.3.2 Sporozoites and merozoites

The mature oocyst undergoes excystation under favorable conditions within the host to release the four infective sporozoites. Merozoites develop in a meront which upon maturation, merozoites are released. Both sporozoites and the merozoites a can invade host cells and are morphologically similar. They are crescent in shape, with the anterior end slightly pointed and the posterior end rounded. In the anterior region, there is an apical complex that is important for locomotion and cell invasion by having apical rings, conoids, rhoptries, micronemes, and electron-dense granules.

1.3.3 Trophozoites

Trophozoites are transitional forms of sporozoites or merozoites to meronts. The crescent shape sporozoites or merozoites approach the anterior end of the host cells for invasion and become spherical to develop the trophozoite stage. Trophozoites are round or oval shaped and vary in size ranging (2.0 to 2.5 μm) and contains a single nucleus with a prominent nucleolus. At the site of electron-dense attachment to the host cells, unique feeder organelle is formed. Also, its apical complex starts to disappear, and vacuoles cluster to form parasitophorous vacuole membrane (PVM) during invasion. This membrane is formed by extensions from host cell cytoplasmic membrane to surround the parasite which is not in contact with the host cell cytoplasm. Hence, it is intracellular but extra cytoplasmic [17, 40, 41].

1.3.4 Meronts

The trophozoites will undergo asexual phase of reproduction (merogony) to form multinuclear meronts. The type I meronts are first generation of meronts from which the merozoites are released become extracellular to re-infect new host cells. Then, they develop into either type I or type II meronts which are are morphological similar (4-5 μm in diameter), except that type I meront has 6 or 8 merozoites, while a type II meront typically has 4 merozoites. Type I merozoites may enter one or more cycles of mergony before developing into type II meronts . During maturation, merozoites become more elongated and develop more micronemes, rhoptries, electron dense granules and ribosomes in the cytoplasm [18].

1.3.5 Gamonts and gametes (sexual stages)

Type II merozoites invade new host cells and undergo sexual development to form microgamonts (male)and microgamonts (female)[40]. A microgamont has a very compact nucleus and lacks rhoptries, unlike the merozoites [18](16). Mature microgamont may contain up to 16 bullet-shaped microgametes which are rod shaped lacking flagella and mitochondria, unlike other coccidia. It also has a slightly falttened anterior end with condensed nucleus. A mature macrogamont contains only one macrogamete which has a large nucleus, prominent nucleolus and a feeder organelle [18, 36].

1.4 Life cycle

The complicated life cycle of *Cryptosporidium* species involves both sexual and asexual phases within a single host. The sporulated oocyst is the infective stage that is excreted in the host feces [17, 40] as shown in ***Figure 1***.

The infection is acquired through the ingestion of food or water containing the occysts by the susceptible host. Suitable conditions are required for excystation of sporozoites, such as appropriate stomach reducing conditions, temperature, pancreatic enzymes, and bile salts [42, 43]. Excystation was demonstrated in aqueous solutions in experimental conditions which may assist in autoinfection and extra-intestinal infections[17, 34]. Excystation of oocysts release the motile sporozoites through an open suture in the oocyst wall [36].The slender shaped motile sporozoite will rapidly attach to the apical end of the intestinal cells and become a spherical-shaped trophozoite. During invasion, vacuoles are formed to surround the parasite and form pre-parsitophorous vacuole which then fuses with the host cell membrane to form the paarsitophorous vacuole (PV) [36, 44, 45] [46]. Asexual reproduction, also called merogony, begins when the trophozoite nucleus divides to form type I meront containing 8 to 16 haploid merozoites, first generation of merogony. The type I merozoites infect other host cells to enter second generation of merogony, forming type II meront containing four merozoites. Type II merozoites invade new host cells and initiate sexual reproduction in which they differentiate in to microgamonts (male) or macrogamonts (female) are formed. Fertilization between micro- and macro-gametes forms the diploid cells, zygotes. A zygote immediately undergoes sporogony to form an oocyst containing four haploid sporozoites. Most of the oocysts (80%) of the oocysts have a thick, two-layer wall and released into the environment while the remaining (20%) are thin, one layer walled. The thin walled oocysts can easily rupture within the same host and release sporozoites for auto-infection. Also, they may have role in persistent and chronic infections in immunosuppressed patients [18, 47].

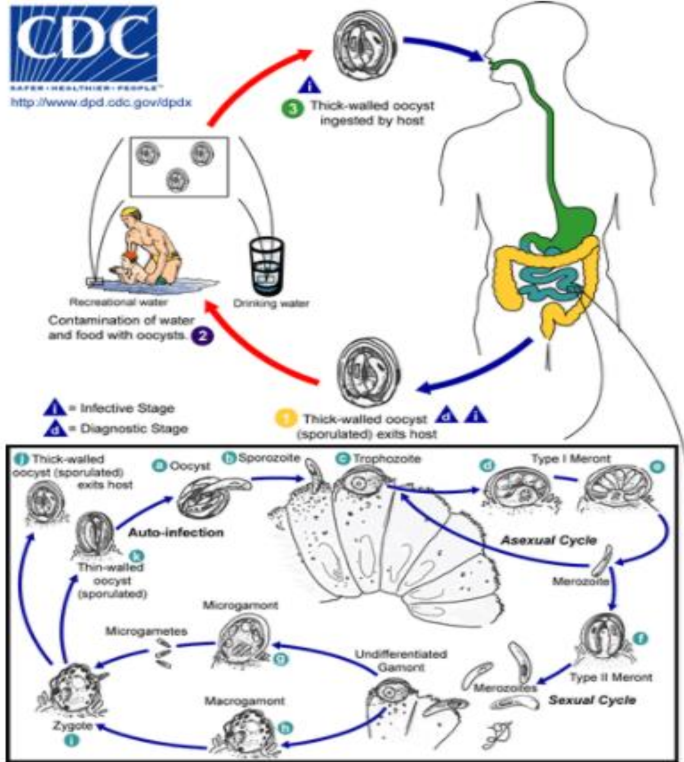

Figure 1 Life cycle of *Cryptosporidium parvum*

(Reproduced from *https://www.cdc.gov/parasites/crypto/pathogen.html)*

Sporulated oocysts, containing 4 sporozoites, are excreted by the infected host through feces and possibly other routes such as respiratory secretions ①. Transmission of *Cryptosporidium parvum* and *C. hominis* occurs mainly through contact with contaminated water (e.g., drinking or recreational water). Occasionally food sources, such as chicken salad, may serve as vehicles for transmission. Many outbreaks in the United States have occurred in waterparks, community swimming pools, and day care centers. Zoonotic and anthroponotic transmission of *C. parvum* and anthroponotic transmission of *C. hominis* occur through exposure to infected animals or exposure to water contaminated by feces of infected animals ❷. Following ingestion (and possibly inhalation) by a suitable host ❸, excystation ⓐ occurs. The sporozoites are released and parasitize epithelial cells (ⓑ, ⓒ) of the gastrointestinal tract or other tissues such as the respiratory tract. In these cells, the parasites undergo asexual multiplication (schizogony or merogony) (ⓓ, ⓔ, ⓕ) and then sexual multiplication (gametogony) producing microgamonts (male) ⓖ and macrogamonts (female) ⓗ. Upon fertilization of the macrogamonts by the microgametes (ⓘ), oocysts (ⓙ, ⓚ) develop that sporulate in the infected host. Two different types of oocysts are produced, the thick-walled, which is commonly excreted from the host ⓙ, and the thin-walled oocyst ⓚ, which is primarily involved in autoinfection. Oocysts are infective upon excretion, thus permitting direct and immediate fecal-oral transmission.

1.5 Cryptoporidiosis

Cryptosporidium parasites are the causative agents of cryptosporidiosis in humans and animals [48-50]. Cryptosporium typically infects epithelial cells in the microvillus border of the gastrointestinal tract of hosts causing severe watery diarrhea. *C. parvum* and *C. hominis* are the two major human pathogens. According to the global enteric multicenter study (GEMS), *C. parvum* and *C. hominis* are among the top two (or top four) diarrheal pathogens in children under the age of one (or age of five) in developing countries. These diarrheal pathogens not only impede the growth and cognitive fitness of children but are also associated with fatality in toddlers aged 12–23 months [51-53]. Additionally, Cryptosporidium can cause opportunistic infection with prolonged, life threatening diarrhea in AIDS patients but mostly self-limited in immunocompetent patients [48, 49].

1.6 Immune responses

The host immune system is necessary to control and eliminate *Cryptosporidium* infections which rely on the host immune system. Cryptosporidiosis is usually self-limiting in immunocompetent individuals but can be persistent and fatal in immunocompromised patients as AIDS patients. There are still gaps in understanding the mechanism responses. Both innate and adaptive immune responses in hosts are important to controlling the disease but the innate immunity has the major contribution in clearing the infection [54, 55]. Here we will highlight the major components of the immune response to cryptosporidial infections in different hosts.

1.6.1 T cells (CD4$^+$ and CD8$^+$ T cells)

The adaptive response is stimulated by specific antigens recognized by T-cells and

B-cells and amplifies the elements of the innate immune response. $CD4^+$ T-cells are activated by the antigen presenting cells (macrophages and dendritic cells) which in turn stimulate the B-cells to proliferate and produce antibodies [56]. In cryptsporidial infections, the T cell immune response relies mainly on $CD4^+$ T-cells rather than on $CD8^+$ T cells. The protective role of $CD4^+$ T cells is evident in several mice studies. Wild type mice were able to eliminate the infection within 8 weeks ,unlike the MHC class II deficient mice lacking CD^+ 4 cells [57] . Consistently, mice infected with *C.muris* and treated with anti- $CD4^+$ antibodies to deplete the $CD4^+$ T cells showed exacerbated infections [58]. Also, lymphocytes from immunocompetent mice were injected into SCID mice, thus providing protective effects against cryptosporidium infection until CD^+ 4 cells were depleted. [58] [59, 60]. The role of $CD8^+$ T cells is still debatable. MHC class I deficient mice lacking show comparable levels of infection with wild type [57]. Added to this, SCID mice lacking $CD8^{+8}$ cells could not eliminate the infection when provided with spleen cells of immunocompetent mice [59]. On the other hand, another study showed SCID could not overcome the infection when supplied with donor spleen cells [58]. Also , treatment of immunocompetent mice with anti- $CD8^+$ had no effect on controlling the infection but the combination of both , anti- $CD8^+$ and anti- $CD4^+$antibodies exacerbates the infection [60]. Moreover, studies in human were conducted showing the dependence of the clinical outcome on the $CD4^+$ cell count. The $CD4^+$ cell counts over 180 cells/µl, cryptosporidiosis is generally self-limited in human but infection is life-threatening in patients (as AIDS patients) with lower $CD4^+$ cells counts (<50 cells/µl) [54, 61](72,74). Additionally, HIV-infected patient was cleared from cryptosporidiosis by the rapid increase in $CD4^+$ cells during highly active antiretroviral therapy [62]. These results support the significance of

$CD4^+$ cells in immune response towards cryptosporidium infections with moderate role $CD8^+$ cells.

1.6.2 B cells

The roles of B cells and antibodies in cryptosporidiosis immune response are not yet clear. B cell deficient mice show comparable susceptibility to infection with the wild type [63]. Another study showed no change in the protective effect in SCID mice treated with spleen cells lacking B cells compared with mice treated with spleen cells that are not depleted of B cells [64]. Also, mice lacking B cells follow similar pattern of infection and clearance as the control mice [65].

On the other hand, studies to understand the role of antibodies in immune response were contradictory. A relation between selective IgA deficiency and persistent cryptosporidial infection was studied supporting the role of B cells in the immune response [66]. Patients with congential hypogamma globulinemia fail to clear the parasite infection [5]. The high serological responses to a 27-kDa *Cryptosporidium*-antigen was accompanied with a reduced risk of diarrhea in both HIV-negative and HIV-positive patients [67]. Also, in immunocompetent patients infected with *C. parvum,* the level of secretory IgA was elevated in patients excreting oocysts or with diarrheal symptoms [68] . Oral treatment of rodents with secretory IgA provided passive protection against infection [67]. On the contrary, *Cryptosporidium*-specific antibody levels are probably unrelated to the diarrheal symptoms [69]. High levels of parasite-specific antibodies (secretory IgA) were noticed in AIDS patients with chronic cryptosporidiosis [70]. Experiments with B- cell deficient mice showed comparable susceptibility to *C. parvum* infection compared with the control group [63] . In a *C.muris* study,

SCID mice treated with splenic cells depleted of B-cells similar pattern of infection as the group not receiving depleted B-cells [64]. Therefore, B cells and antibodies may have role in protection against infection but may not be crucial as other factors.

1.6.3 IFN-γ

IFN-γ is a Th1 cytokine that plays a significant role in the adaptive response towards cryptosporidial infections. Treatment of SCID and immunocompetent mice with splenic cells and anti-IFN-γ neutralizing antibodies enhanced the growth of the parasite [64, 71].Also, $CD4^+$ T-cells purified from *C. parvum* immunized mice could proliferate and produce IFN- γ upon incubation with *C. parvum* antigen [72]. *C. parvum* infections were more exacerbated in IFN-γ knockout SCID mice than SCID mice [73](90). Also, the infection was exacerbated when mice were treated with both, anti- $CD4^+$ and anti-IFN-γ antibodies more significantly than with either. This supports the relation between $CD4^+$ T-cells and IFN-γ in the immune response to *C. parvum.* The early and significant increase in ileal IFN-γ content is associated with improved outcomes and recovery from *C. parvum* infection was observed in humans, mice and calves [74-77]. Not only, the IFN-γ mRNA and protein expression was induced by *C. parvum* [78] [74] [79] but also has a pattern similar to the pattern of acute infection [74]. As a therapeutic agent, IFN-γ treatment was able to diminish the parasite burden in immunosuppressed rat [80]. However, it did not provide a protective effect against the infection in immunodeficient mice [81, 82].

1.6.4 IL-12

Interleukin 12 (IL-12) is a cytokine released by the antigen presenting cells

(macrophages and dendritic cells) to induces NK cells and T cells to produce INF-γ in *C. parvum* infection [83]. Not only the treatment of immunocompetent mice with IL- 12 prior to the inoculation with *C. parvum* oocysts provided protection form the infection but also the treatment with anti-IL-12 antibodies exacerbated the infection [78]. IL-12 protection was also associated with IFN-γ production. In which the IL-12 treatment increased the levels of intestinal IFN-γ mRNA which is also related to early control of infection [79]. Despite of the increase in T cells count and expression of INF-γ, the treatment with IL-12 prior to infection in calves did not stimulate adequate resistance [84]. *C. parvum* infections ,with as low as 100oocysts ,would result in acute diseases in the IL-12 knockout adult C57BL/6 mice but were able to recover within 2 weeks [85] . Therefore, IL-12 knockout C57BL/6 mice is used as a model for testing drug efficacies against acute cryptosporidiosis.

1.7 Treatments

The outbreak of cryptosporidiosis Milwaukee in 1993 is the greatest water born outbreak documented in the history of the United States. Together with the subsequent outbreaks worldwide, attention was given to cryptosporidium by the public, government and environmental agencies [11]. New drugs are urgently needed against this pathogen for which various in vitro, in vivo and clinical studies were conducted. However, there is no fully effective drug against cryptosporidiosis up to date. Currently, nitazoxanide (NTZ) is the only FDA approved to treat cryptosporidiosis in immunocompetent patients, but its efficacy is still questionable. Here, we will briefly review the current therapeutic agents with anti-cryptsporidial activities.

1.7.1 Nitazoxanide

Nitazoxanide is the only FDA-approved drug for treatment of cryptosporidiosis in immunocompetent individuals but not in immunocompromised individuals. In an earlier study, three days course of 500 mg of nitazoxanide for adults or 10 mg for children (1-11 years) twice daily significantly decreased diarrhea symptoms and oocyst shedding in immunocompetent patients compared with placebo (114). Among AIDS patients, nitazoxanide was also effective against *C. parvum* in AIDS patients with less diarrheal symptoms [86]. NTZ and its metabolites, tizoxanide (TZ) and tizoxanide- glucuronide (TZglu) inhibited the parasite growth at less than 10 mg/ml [87, 88]. No safety issues were documented up to 3000mg/day or with long duration of NTZ treatment [89].

1.7.2 Paromomycin

Paromomycin is an aminoglycoside with broad spectrum of antimicrobial activity, which is mainly used to treat *Giardia* and amoebic intestinal infectious. It is poorly absorbed from the gut epithelium with oral treatment. It acts by targeting the aminoacyl tRNA site of the ribosomes, thus disrupting the protein synthesis. The anti-cryptosporidial activity of paromomycin was reported in various studies [90-92] including its efficacy in HIV-infected patients [93]. Another study was showed significant parasitological cure by paromomycin among AIDS patients infected with cryptosporidiosis [94]. On the contrary, a study showed paromomycin is not more effective than placebo in an AIDS patients with cryptosporidial infection [95]. Paromomycin is not approved by the FDA drug for treatment of cryptosporidiosis but it is used to alleviate the symptoms. In AIDS patients, it is not recommended to be used unless with HAART (Highly-active anti-retroviral treatment) [96].

1.7.3 Highly-active anti-retroviral therapy

The improvement in the immune system of HIV patients is the key for treatment of cryptosporidiosis. This is achieved by the highly-active anti-retroviral therapy (HAART) that can improve the outcome of opportunistic infections including cryptosporidiosis [96]. HAART can improve cryptosporidiosis symptoms with total parasitological cure in AIDS patients. It is also associated with increase in the $CD4^+$ T-cells which have important role in the immune response against the parasite as mentioned earlier [97]. However, some case control studies reported during the initial treatments [98]. Protease inhibitors (ritonavir, saquinavir and indinavir) were also studied as anti-cryptosporidial agents in which they show dramatic reduction in the parasite loads in both, *in vitro* and *in vivo* models [99, 100].

1.7.4 New thiazolide derivatives

Modifications in the chemical structure of nitazoxanide were performed to synthesize new NTZ derivatives. Romark Center for Drug Discovery has synthesized 42 thaizolides, among which, 26 inhibited *C. parvum* growth invtro by more than 95%. Added to this, all the derivatives with chlorine group instead of the nitro group and most of the bromo thiazolodes showed more than 95 % inhibition on the parasite growth in vitro [101].

1.8 Energy metabolism in *Cryptosporidium*

The whole genomes of *C. parvum* has been sequenced and broadens our knowledge about *C. parvum* metabolism. *Cryptosporidium* are unable to synthesize most nutrients de novo, including amino acids, nucleotides and fatty acids Unlike other apicomplexans, *C. parvum* has a

highly streamlined metabolism and lacks many metabolic pathways such as mannitol cycle, shikimate pathway and electron transport chains, and also has pathways that are highly divergent from other apicomplexans such as the inositol monophosphate dehydrogenase (IMPDH) [13, 14]. This explains the limited classical drug targets in *C. parvum* and its insensitivity towards many drugs that are usually effective against other apicomplexans. *C. parvum* lacks the genes encoding the Krebs cycle along with the apicoplast and mitochondrial genomes that are found in other apicomplexans [19]. Although *C. parvum* may possess a remnant mitochondrion, it lacks Krebs cycle and cytochrome-based respiration, thus mainly, if not only, depending on glycolysis for ATP production [13, 102]. Within the glycolytic pathway, *C. parvum* obtains glucose and other hexoses either directly from the host or through degradation of polysaccharides [103, 104]. To enter metabolic pathways, glucose needs to be activated by hexokinase (HK) to form glucose-6-phosphate (glucose-6P) that will be further converted to fructose-6-phosphate (fructose-6P) by glucose-6P isomerase (GPI), and then to fructose-1,6-pyrophosphate by pyrophosphate-dependent phosphofructokinase (PPi-PFK) before being split into two triose-phosphate molecules. Similar to other microaneorobic protists as *Giardia* and *Trichomonus*, *C. parvum* has pyrophosphate-dependent phosphofructokinase (PPi-PFK), thus generating 3 ATP molecules rather than 2 ATP molecules by human and other aerobic organisms that use the ATP dependent PFK [105]. Phosphoenol pyruvate (PEP) is then converted to pyruvate by pyruvate kinase (PK) releasing one ATP molecules or through malate dehydrogenase (MDH). Pyruvate is converted to acetyl-CoA by the bifunctional pyruvate:$NADP^+$ oxidoreductase (PNO) which has pyruvate ferrodoxin oxidoreduactase (PFO) domain fused with the cytochrome P450 . This is a unique structure in Cryptosporidium that differs from the pyruvate dehydrogenase (PDH) complex in other apicomplexans and in humans and animals but also found in Euglena gracillis [106] . Three

organic end products are produced from Acetyl-CoA which are lactate by lactic acid dehydrogenase (LDH), ethanol by aldehyde dehydrogenase (AdhE) and actetate by acetyl CoA ligase (AceCL). All apicomplexans LDH and MDH including CpLDH and CpMDH are acquired from α- proteobacterial ancestor [107, 108]. Besides, CpMDH and other apicomplexans have one cytosolic MDH unlike other eukaryptes which have several MDH isoenzymes (cytosolic, mitochondrial and apicoplastic) [108]. The degradation of polysaccharides give rise to glucose-1P which can give rise to glucose-6P by glucomutase enzyme. Unlike other apicomplexans, Glucose-1P and UDP glucose can be used by *C. parvum* in the synthesis of an antistress molecule, trehalose. It is an anti-oxidant and protein stabilizing agent that is synthesized in some bacteria and plants [109, 110]. Thus, suggesting the possibility of using trehalose as a source of energy, however, other enzymes involved in its metabolism are not yet revealed.

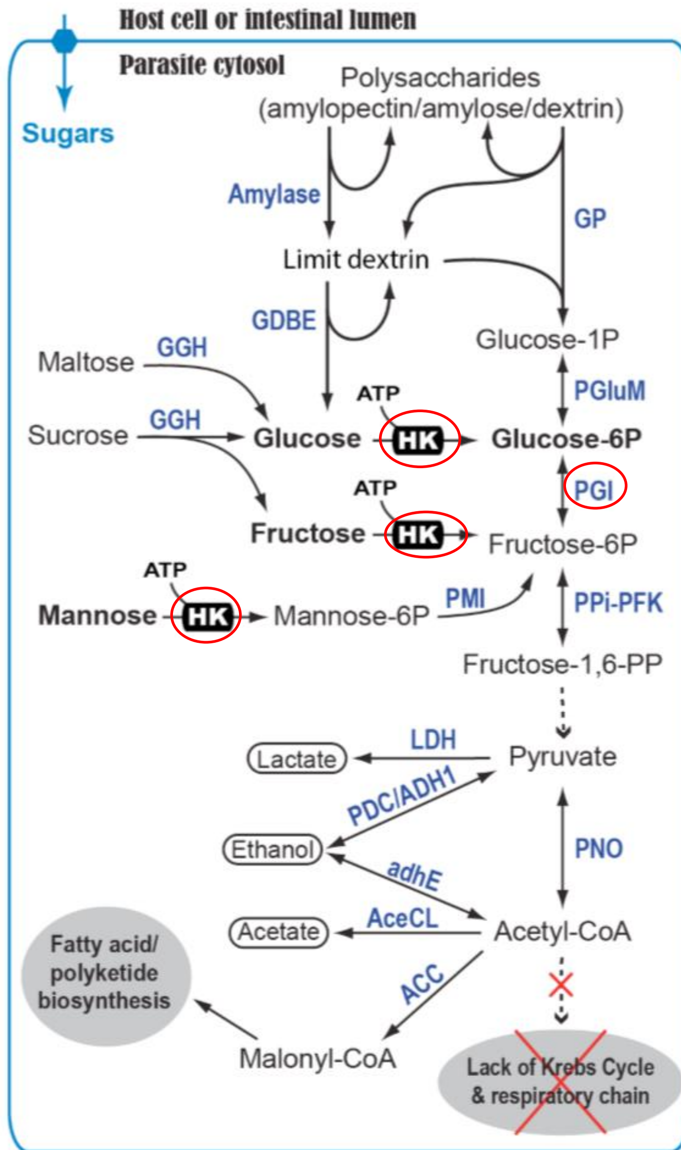

Figure 2 The role of CpHK and CpGPI in the unique glycolytic pathway and major connections in *Cryptosporidium parvum* [104]

Abbreviations: **ACC**, acetyl-CoA carboxylase; **AceCL**, acetic acid-CoA ligase; **ADH1**, alcohol dehydrogenase 1; **adhE**, type E alcohol dehydrogenase (bifunctional); **GDBE**, glycogen debranching enzyme; **GGH**: Glucoside glucohydrolase; **GP**: glycogen phosphorylase; **HK**, hexokinase; **LDH**, lactate dehydrogenase; **PGI**, phosphoglucose isomerase; **PGluM**, phosphoglucose mutase; **PMI**: phosphomannose isomerase; **PDC**: pyruvate decarboxylase; **PNO**, pyruvate:$NADP^+$ oxidoreductase; **PPi -PFK**: pyrophosphate-dependent phosphofructokinase.

2 EXPLORING GLUCOSE -6-PHOSHPHATE ISOMERASE (GPI) AS A POTENTIAL DRUG TARGET IN *CRYPTOSPORIDIUM PARVUM*[*]

2.1 Overview

Cryptosporidium parasites are the causative agents of cryptosporidiosis in humans and animals [49, 111, 112]. According to the global enteric multicenter study (GEMS), *C. parvum* and *C. hominis* are among the top two (or top four) diarrheal pathogens in children under the age of one (or age of five) in developing countries. These diarrheal pathogens not only impede the growth of children, but are also associated with fatality in toddlers aged 12–23 months [51, 113, 114]. Additionally, *Cryptosporidium* can cause opportunistic infection with prolonged, life-threatening diarrhea in AIDS patients [49, 112]. Up to date, nitazoxanide is the only FDA-approved drug in the United States to treat cryptosporidiosis in immunocompetent patients, while there are no effective and safe treatments for cryptosporidiosis in immunocompromised patients. Also, there is no approved treatment for cryptosporidiosis in animals in the United States [49, 115, 116]. Therefore, there is a critical need for development of new anti-cryptosporidial drugs. Unlike other apicomplexans, *C. parvum* has a highly streamlined metabolism and lacks many metabolic pathways such as mannitol cycle , shikimate pathway and electron transport chains, and also has pathways that are highly divergent from other apicomplexans such as the inositol monophosphate dehydrogenase (IMPDH) [13, 117]. This explains the limited classical drug targets in *C. parvum* and its insensitivity towards many drugs that are usually effective against other apicomplexans. *C. parvum* lacks the genes encoding the Krebs cycle along with the

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[*] Parts of this section are reprinted from Rana Eltahan , Fengguang Guo , Haili Zhang , Lixin Xiang , Guan Zhu, Discovery of ebselen as an inhibitor of *Cryptosporidium parvum* glucose-6-phosphate isomerase (CpGPI) by high-throughput screening of existing drugs. International Journal for Parasitology: Drugs and Drug Resistance, *Volume 8, Issue 1, April 2018. Article is Open Access and available online at:* https://doi.org/10.1016/j.ijpddr.2018.01.003

apicoplast and mitochondrial genomes that are found in other apicomplexans [19]. Although *C. parvum* may possess a remnant mitochondrion, it lacks Krebs cycle and cytochrome-based respiration, thus mainly, if not only, depending on glycolysis for ATP production [13, 102]. The glycolytic enzymes, many of which are highly divergent from humans and animals, may therefore be explored as potential drug targets.

Within the glycolytic pathway, *C. parvum* obtains glucose and other hexoses either directly from the host or through degradation of polysaccharides [103, 104]. To enter metabolic pathways, glucose needs to be activated by hexokinase (HK) to form glucose-6-phosphate (glucose-6P) that will be further converted to fructose-6-phosphate (fructose-6P) by glucose-6P isomerase (GPI), and then to fructose-1,6-pyrophosphate by pyrophosphate-dependent phosphofructokinase (PPi-PFK) before being split into two triose-phosphate molecules (**Fig. 1A**).

In the present study, we characterized the molecular and biochemical features of the GPI in *C. parvum* (CpGPI) encoded by a single-copy *CpGPI* gene. We also screened a Prestwick chemical library containing 1200 known drugs for potential anti-CpGPI activities and discovered that ebselen could inhibit CpGPI. We observed that ebselen inhibited the activity of CpGPI more effectively than that of human (*Homo sapience*) GPI (HsGPI), and that the growth of *C. parvum* in vitro could be inhibited by ebselen at levels nontoxic to the host cells.

2.2 Materials and methods

2.2.1 Molecular cloning of CpGPI gene and expressionof recombinant CpGPI protein

The oocysts of *C. parvum* (Iowa-1 strain) were purchased from Bunch Grass Farm (Deary, ID) and experiments used oocysts that were less than three months old since harvest. Oocysts

were purified from calf feces by a sucrose-gradient centrifugation, followed by treatment with 10% Clorox on ice for 7 minutes and then washed 5 – 8 times in pure water [118]. Oocysts were further purified by a Percoll gradient centrifugation protocol and finally suspended in phosphate-buffered saline (PBS; pH7.2) and stored at 4°C before use.

CpGPI gene has been annotated by the *C. parvum* genome-sequencing project (Gene ID: cgd2_3200; GenBank: XP_626511). The open reading frame (ORF) was amplified from the *C. parvum* genomic isolated from oocysts with a DNeasy Blood & Tissue Kit (Qiagen) by PCR using a high-fidelity *Pfu* DNA polymerase with the primer pair of CpGPI-F-BamHI (5′ AGG GAT CCA TGC CAG AAC TTT ATG AAC 3′) and CpGPI-R-SalI (5′ ATG TCG ACA TTC GTC AGG CTC TTT GAA 3′). The amplicons were ligated into a pCR2.1-TOPO vector (Invitrogen) linearized by *Bam*I and *Sal*I, followed by transfection into NEB 5-alpha *Escherichia coli* cells (New England BioLabs). Plasmids were isolated from several bacterial colonies using an E.Z.N.A. Plasmid Mini Kit I (Omega Bio-tek; Norcross, GA), and sequenced to confirm their identities and sequence accuracy. The DNA fragment containing the *CpGPI* ORF was then subcloned into a pMAL-c2E-TEV-His plasmid derived from the pMAL-c2E plasmid, which was engineered to encode a tobacco etch virus (TEV) cleavage site between the N-terminal MBP-tag and C-terminal fusion protein, together with a His-tag at the C-terminus of recombinant protein [119]. The expression of CpGPI as an MBP-fusion protein (MBP-CpGPI) was carried out in the Rosetta-2 strain of *E. coli* competent cells (EMD Millipore; Burlington, MA). The induction of expression and the purification of MBP-fusion proteins using amylose resin-based affinity chromatography followed standard procedures [119]. The quality and the quantity of recombinant MBP-CpGPI protein were evaluated by SDS-PAGE and Bradford protein assays using bovine serum albumin (BSA) as a standard.

2.2.2 Biochemical assays and high-throughput screening of known drugs

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or as specified. The glucose-6P dehydrogenase (G6PDH)-coupled assay was used to evaluate the CpGPI enzyme activity and kinetics by a reverse reaction that converted fructose-6P into glucose-6P by CpGPI and then converted glucose-6P into glucono-1,5-lactone-6-phosphate by G6PDH using NAD^+ as a cofactor (**Fig. 1A**). A typical assay was performed using 200 µL reactions containing D-fructose-6P (2 mM), NAD^+ (0.2 mM), G6PDH (2 U), $MgCl_2$ (5 mM) in Tris.HCl buffer (50 mM, pH8.5), and MBP-CpGPI or MBP (50 ng). The reaction started with the addition of MBP-CpGPI (or MBP). The production of NADH was monitored spectrophotometrically at 340 nm every min for up to 30 min in a Multiscan Spectrum spectrophotometer (Thermo Scientific; Waltham, MA). MBP-tag only was used in all assays as negative control and for background subtraction.

The G6PDH-coupled assay was employed to screen the Prestwick chemical library containing 1,200 known drugs (http://prestwickchemical.com) to identify potential anti-CpGPI activities. Each reaction in the primary screening was performed at least twice independently in the presence of 20 µM compounds at room temperature. Each 96-well plate contained two triplicated negative controls including standard reactions without CpGPI, but containing MBP-tag, and reactions containing CpGPI and diluent (1.0% DMSO), and a positive control using erythrose-4 phosphate (erythrose-4P) at 0.2 mM.

After ebselen was identified as the greatest inhibitor, another assay was performed to confirm that the effect observed in the CpGPI/G6PDH-coupled assay was attributed to the inhibition of ebselen on CpGPI, rather than to that of G6PDH. This assay used glucose-6P as the substrate in the absence of CpGPI and F6P. Lowered levels of G6PDH (0.04 U) and glucose-6P (0.2 mM)

were used to minimize the potential masking of the inhibitory effect on G6PDH. A more detailed analysis was performed to determine the IC_{50} value of ebselen on CpGPI. MBP-tag only was used in all assays as a negative control and for background subtraction. The effect of ebselen on the enzyme activity of human GPI (HsGPI) (purchased from Biovision; Milpitas, CA) was similarly carried out for comparison. All assays were performed at least twice in triplicated reactions at room temperature (23 °C). Enzyme kinetic data were analyzed using GraphPad Prism v5.0f (http://www.graphpad.com) with appropriate models.

The reversibility of the inhibition of ebselen on CpGPI was tested by an inhibitor dilution assay. 100 µM of ebselen was pre-incubated with 100 ng of CpGPI in a volume of 4.0 µL on ice for 10 min, followed by the addition of other reaction components to reach a final concentration of ebselen at 2 µM in a 200 µL reaction (i.e., 50-fold dilution of ebselen). Whether the reducing agent dithiothreitol (DTT) could block the inhibition of ebselen on CpGPI was evaluated by pre-incubating DTT with CpGPI in a volume of 3.0 µL on ice for 10 min prior to the addition of other reaction components (including ebselen). Whether DTT could reverse the drug inhibition was evaluated by pre-incubating CpGPI with ebselen on ice for 10 min prior to the addition of other reaction components (including DTT). In the two DTT assays, the final reaction volume was 200 µL containing CpGPI (100 ng), ebselen (100 µM), and DTT (100 mM). Reactions containing DTT without pre-incubation were included in both assays as a control.

2.2.3 Drug efficacy against the parasite growth in vitro

A quantitative real-time RT-PCR (qRT-PCR) assay developed in our laboratory was used to evaluate drug efficacy against the growth of *C. parvum* in vitro [87, 120]. Briefly, HCT-8 cells (ATCC # CCL-225) were seeded in 96-well plates in RPMI-1640 medium containing 10% fetal bovine serum (FBS), and cultured at 37 °C under 5% CO_2 atmosphere overnight or until they

reached to between 80% – 90 % confluence. Fresh *C. parvum* oocysts were purified as described [87, 120], and were added to the host cell monolayers with a parasite to host cell ratio at 1:2 (i.e., 2×10^4 oocysts with ~4×10^4 host cells per well). After incubation for 3 h at 37 °C, during which time the parasites underwent excystation, the medium was exchanged to remove the free parasites. Infected cells were incubated for an additional 41 h (total 44 h infection time) with ebselen at specified concentrations.

The microplates were centrifuged at 1,000 × *g* for 10 min in a plate centrifuge (Heraeus Megafuge 16; Thermo Scientific, Waltham, MA) to ensure that all cells, including merozoites,that could be released into the medium were firmly attached at the bottom of the wells. Cells were gently washed at least 3 times with 200 µL PBS, followed by the addition of 150 µL/well of ice cold iScript RT-qPCR sample preparation reagent (Bio-Rad Laboratories; Hercules, CA). The plates were then sealed with adhesive sealing films and placed in a bucket containing ice to be vortexed for 20 min in a VX-2500 orbit shaker (VWR International; Radnor, PA), followed by incubation at 75 °C for 15 min, and finally centrifuged (5 min, 2,000 x *g*) to separate supernatants from cell debris. The supernatants were used immediately for qRT-PCR or stored at -80 °C for future use.

A qScript One-Step SYBR Green qRT-PCR kit (Quanta biosciences; Gaithersburg, MD) was used to detect the relative levels of parasite 18S rRNA (Cp18S) and human 18S rRNA (Hs18S). The relative parasite loads were determined by first calculating the ΔC_T values between the Cp18S and Hs18S in individual samples and then $\Delta\Delta C_T$ between experimental samples and controls as described [87, 120-122]. The IC_{50} values of inhibitors were analyzed using GraphPad Prism version 5.0 (http://www.graphpad.com). Paromomycin (100 µg/mL) and diluent (0.5% DMSO) were used as positive and negative controls, respectively. Cytotoxicity of inhibitors on

host cells was monitored by microscopic observation of the cell morphology and detachment, and evaluated by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and by detecting the relative levels of 18S rRNA in uninfected HCT-8 cells.

2.3 Data analysis

All the biochemical assays and in vitro drug assays were performed at least twice and independently. Data was analyzed with Microsoft Excel and GraphPad Prism version 5.0f or higher (http://www.graphpad.com).

2.4 Results

2.4.1 CpGPI enzyme kinetics

CpGPI is a single copy gene with ORF defined by 1,704 nt that can be conceptually translated into 568 aa with an estimated molecular weight of 63.2 kDa and a pI of 8.77. CpGPI protein was successfully expressed as an MBP-fusion protein and purified into homogeneity for evaluating enzyme activity by a GPI/G6PDH-coupled assay (Figure 3A, Figure 3B). The CpGPI enzyme activity was pH-dependent with an optimal value at pH8.5 (***Figure 3***C). CpGPI displayed Michaelis-Menten kinetics towards fructose-6P (K_m = 0.309 mM, V_{max} = 31.72 nmol/μg/min) (Fig. 3D).

2.4.2 Identification of ebselen as a CpGPI inhibitor from 1,200 existing drugs

GPI/G6PDH-coupled assay was used to screen the Prestwick Chemical Library containing 1,200 known drugs for identifying potential CpGPI inhibitors. Erythrose-4-phosphate (erythrose-4P) at 200 μM was used as a positive control. Erythrose-4P was previously reported as a

competitive inhibitor of GPIs from other protozoa (e.g., *Trypanosoma cruzi* and

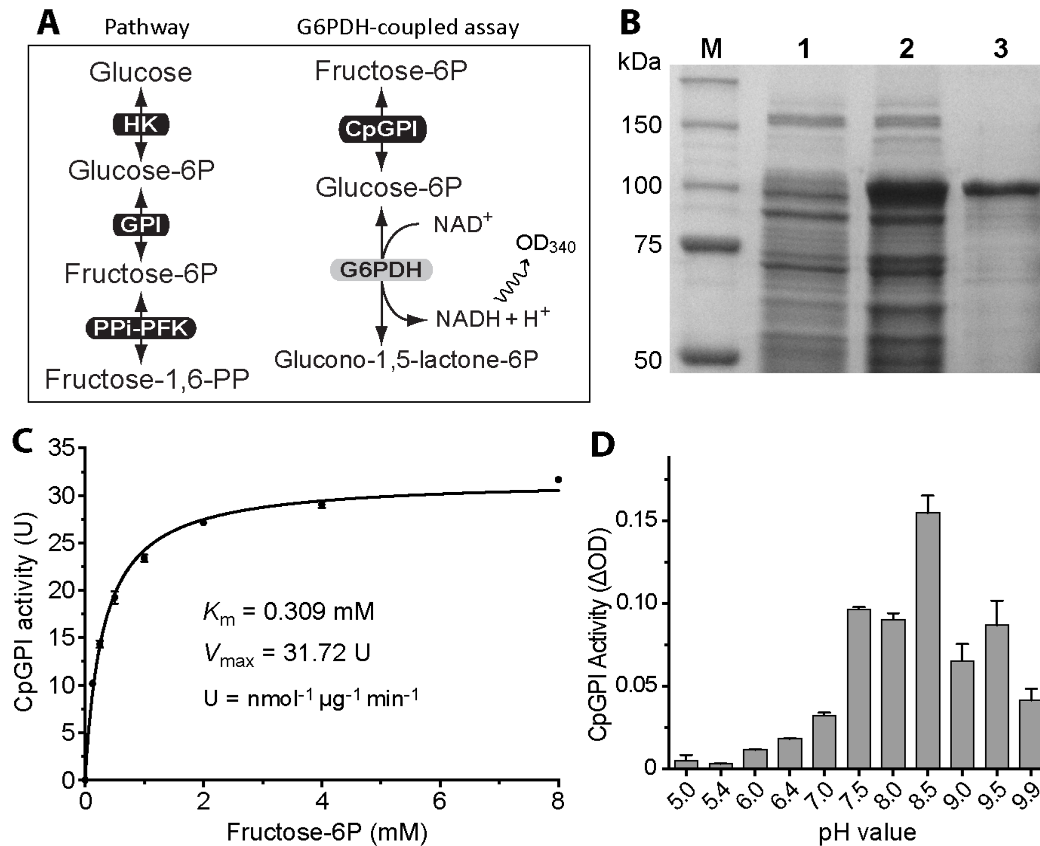


Figure 3 Enzyme kinetic features of CpGPI

A) Illustrations of the reaction catalyzed by GPI and the G6PDH-coupled assay for detecting the CpGPI enzyme activity; **B**) SDS-PAGE gel image showing the expression and purification of recombinant CpGPI as an MBP-fusion protein. Lane M, protein markers; Lanes 1 and 2, total proteins from transformed bacteria before the induction of expression by isopropyl β-D-1-thiogalactopyranoside (IPTG); Lane 3, recombinant MBP-CpGPI protein purified by amylose resin-based affinity chromatography; **C**) Effect of pH on the activity of CpGPI; and **D**) Michaelis-Menten kinetics of recombinant CpGPI towards fructose-6P. Each dataset shown here represents one of the typical experiments performed independently at least three times. Bars represent standard errors of the mean (SEMs) derived from at least three replicated reactions.

Dictyostelium discoideum) [123, 124], archea (e.g., *Pyrobaculum aerophilum*, *Methanococcus jannaschii, Aeropyrumpernix* and *Thermoplasma acidophilum)* [125-127]*, and some other*

species such as plants (e.g., spinach leaf) and fungi (e.g., *Aspergillus Niger*) [128, 129]. In this study, we also observed that erythrose-4P was able to inhibit CpGPI (IC_{50} = 131.5 µM) (**Fig. 4B**).

In the primary screening (20 µM), three compounds (i.e., ebselen, lueolin and merbromin) displayed ≥50% inhibition on the CpGPI enzyme activity (**Fig. 4A**). Merbromin, a toxic organomercuric compound, was excluded from further analysis. Subsequent validation experiments revealed that lueolin was a false-positive because it inhibited G6PDH enzyme activity (i.e., 53.2% and 42.7% inhibition on CpGPI and G6PDH at 20 µM, respectively), but confirmed ebselen as an authentic anti-CpGPI (i.e., 77.1% and 3.7% inhibition on CpGPI and G6PDH at 20 µM, respectively) (**Fig. 4C**). Ebselen (**Fig. 4A, inset**) was the single CpGPI inhibitor identified from the 1,200 existing drugs tested.

2.4.3 Ebselen acted as an allosteric noncompetitive inhibitor on CpGPI

Ebselen had an IC_{50} value of 8.33 µM on CpGPI (**Fig. 5A**). The value was more than 15-fold lower than that of the erythrose-4P. However, 100% inhibition of the CpGPI activity was not achieved by ebselen at concentrations up to 128 µM. The action of ebselen on CpGPI fit with a mixed noncompetitive model of inhibition with cooperativity (**Fig. 5B**). The model used in the nonlinear regression was derived from the equation 3.3 as described by Copeland, but modified with the consideration of Hill coefficient (***n***) [130]

$$v = \frac{V_{max}[S]^n}{[S]^n\left(1+\frac{[I]}{\alpha K_i}\right)+K_m(1+\frac{[I]}{K_i})} \quad (1)$$

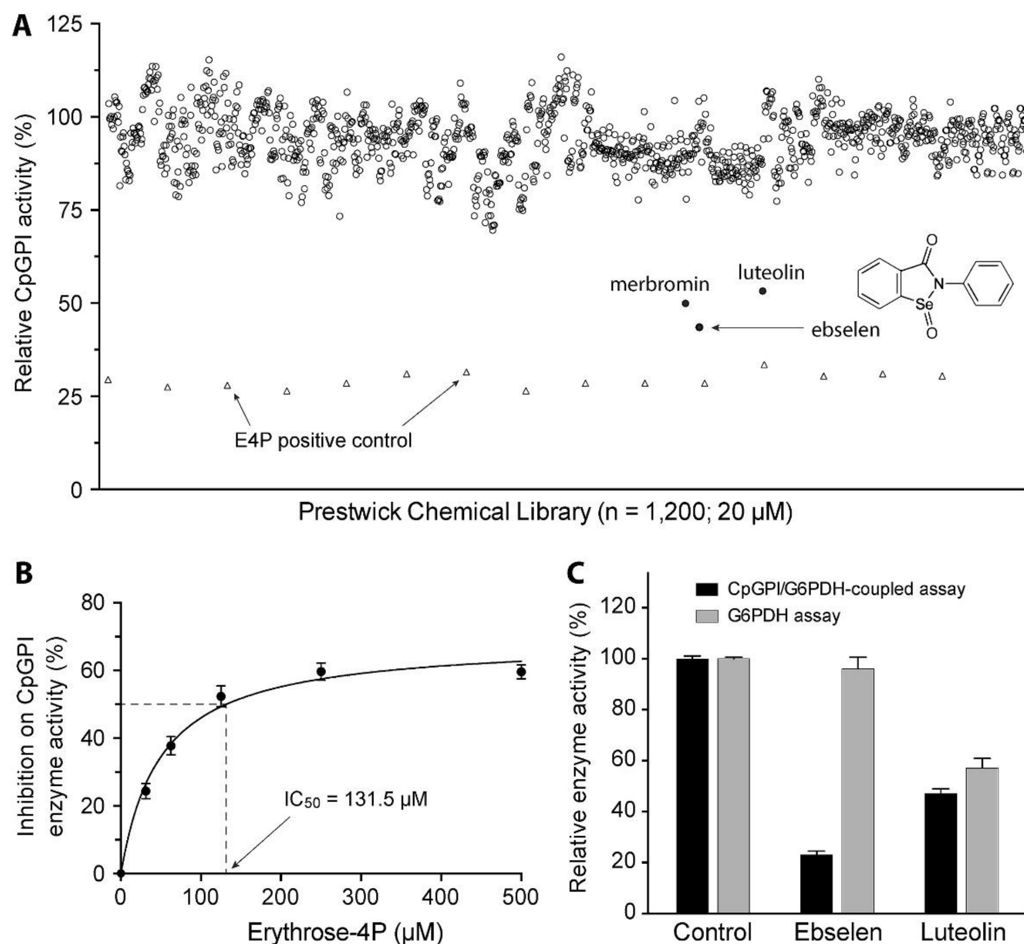


Figure 4 Identification of ebselen as an inhibitor of CpGPI from existing drugs.

A) Screening of the Prestwick chemical library containing 1,200 existing drugs at 20 μM (open round circles). Erythrose-4P at 200 μM was used as a positive control (open triangles). The three top hits are labeled by names, including the chemical structure for ebselen. **B**) Dose-dependent inhibition of CpGPI activity by the positive control erythrose-4P. **C**) Hit validation assay showing that ebselen was an authentic hit because it was ineffective on G6PDH used in the CpGPI/G6PDH-coupled assay, whereas luteolin was a false hit because its inhibition observed in the CpGPI/G6PDH-coupled assay was mainly attributed by inhibition on G6PDH. Bars represent standard errors of the mean (SEMs) derived from at

where $\boldsymbol{v}$ is the enzyme velocity, [$\boldsymbol{S}$] is the substrate concentration, $\boldsymbol{K_i}$ is the inhibitory constant, and $\boldsymbol{\alpha}$ defined as the mode of action. In this study, the K_i value was determined to be 36.33 μM (**Fig. 5B**). The α parameter was determined to be 0.358 (**Fig. 5B**), indicating that ebselen bound to CpGPI with greater affinity then to the enzyme-substrate complex. The $\boldsymbol{n}$ values ranged from 1.065 in the absence of an inhibitor (no cooperativity in enzyme kinetics) to 0.346 and 0.295 in the presence of 1 μM and 5 μM of an inhibitor (negative cooperativity), suggesting that ebselen acted as an allosteric noncompetitive inhibitor of CpGPI.

2.4.4 Ebselen was a selective and irreversible CpGPI inhibitor

Ebselen was a highly selective inhibitor on CpGPI in comparison to the human counterpart, as it was ineffective on HsGPI at concentrations up to 400 μM (vs. IC_{50} = 8.33 μM on CpGPI) (**Fig. 5C**). This inhibitor acted on CpGPI irreversibly, as the inhibition could not be reversed by up to 50-fold dilution of the inhibitor (i.e., from 100 μM to 2 μM) (**Fig. 5D**). Dilution was achieved by pre-incubation of enzyme and inhibitor prior to the addition of other assay components.

A previous study showed that the incorporation of the reducing agent DTT could reverse the ebselen inhibition of human indoleamine-pyrrole 2,3-dioxygenase (IDO) [131]. In this study, DTT could block but not reverse the inhibition of ebselen on CpGPI, as DTT at 100 mM only slightly affected the inhibition when it was incubated with the enzyme prior to or during the reaction (**Fig. 5D**). These observations suggest that, similar to the properties observed for a *Trypanosoma brucei* hexokinase (TbHK1) [132], ebselen might bind to CpGPI covalently (in the absence of a reducing agent), likely via the modification of one or more Cys residues of CpGPI that was resistant to DTT treatment, while the direct interaction of DTT and ebselen could

interfere with the association of the inhibitor with the enzyme.

2.4.5 Ebselen inhibited C. parvum growth at levels nontoxic to host cells in vitro

Using a qRT-PCR-based drug testing assay, it was observed that ebselen was able to inhibit the growth of *C. parvum* in vitro at micromolar levels (EC_{50} = 165 μM) (**Fig. 6A**). Microscopic examination of cell morphology revealed little or no apparent cytotoxicity at the tested concentrations. Cytotoxicity assays by the MTT assay showed cytotoxic EC_{50} values for HCT-8 cells at 700 μM, giving a safety interval (i.e., $EC_{50[host\ cell]}/EC_{50[parasite]}$) of 4.2 (**Fig. 6B**).

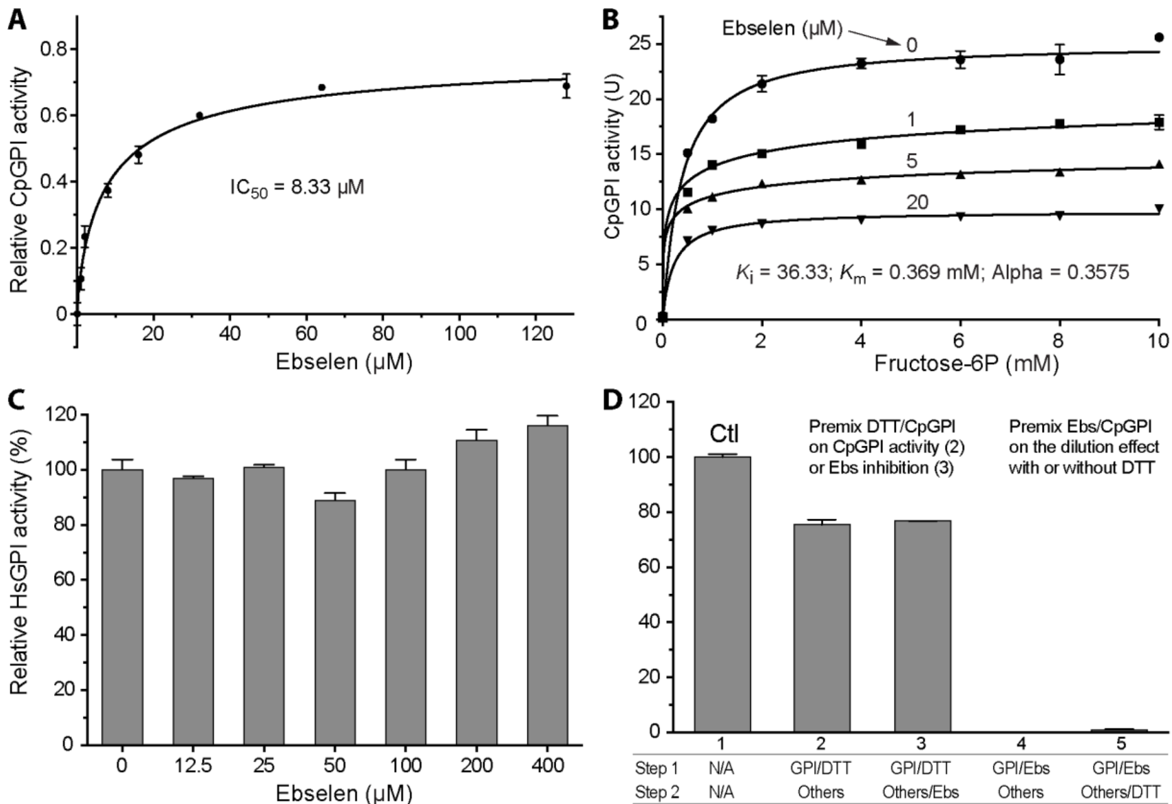


Figure 5 Inhibition of ebselen on CpGPI.

A) Dose-dependent inhibition of ebselen on CpGPI enzyme activity for determining the IC_{50} value; **B)** Allosteric kinetics of CpGPI in the presence of ebselen that fit into a mixed noncompetitive inhibition model; **C)** Effect of ebselen on human GPI (HsGPI) enzyme activity. The assay was performed with ebselen at concentrations up to 400 µM when it reached approximately maximal solubility in the assay; and **D)** Effects of DTT and inhibitor dilution on the inhibition of ebselen (Ebs) on CpGPI. In the DTT assay, pre-incubation of DTT and CpGPI only slightly affected the enzyme activity (lane 2), but blocked the inhibition of ebselen on CpGPI (lane 3). In the inhibitor dilution assay, pre-incubation of ebselen (100 µM) with CpGPI on ice for 10 min followed by 50-fold dilution with the addition of other reaction components had no effect on the inhibition of ebselen on CpGPI with or without DTT (lanes 4-5). Here the final concentration of ebselen in the reactions was

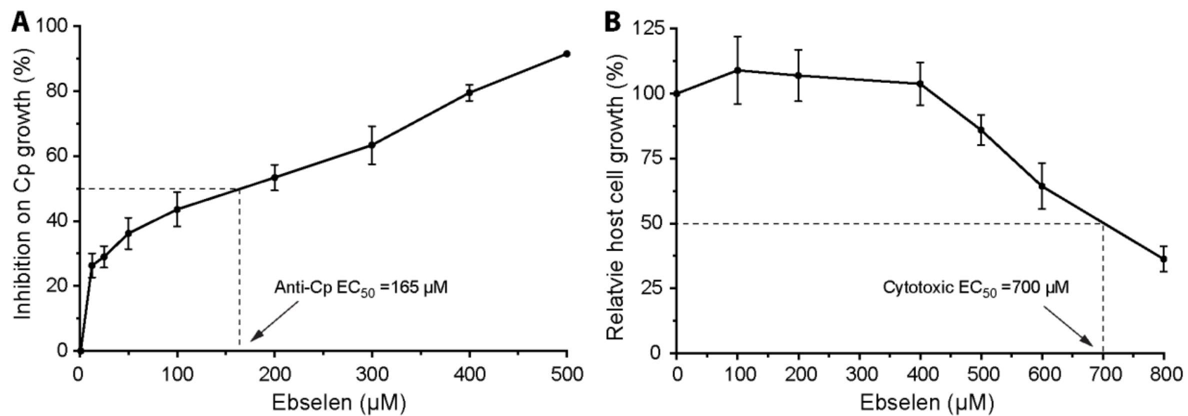


Figure 6 Efficacy of ebselen against *C. parvum* in vitro.

A) Inhibition of ebselen on the growth of *C. parvum* (Cp) cultured in vitro for 44 h as determined by a qRT-PCR assay; and **B)** Cytotoxicity of ebselen when incubated with the uninfected HCT-8 cells for 44 h at specified concentrations and determined by MTT assay. Bars represent standard errors of the mean (SEMs) derived from at least three replicated reactions.

2.5 Discussion

Cryptosporidium parasites rely on glycolysis to produce ATP as they lack the Krebs cycle (intestinal *Cryptosporidium* species only) and cytochrome-based respiration (both intestinal and gastric *Cryptosporidium* species). GPI is the second essential enzyme in the glucose glycolysis, catalyzing the reversible isomerization of D-glucose-6P to D-fructose-6P. GPI was previously studied as a potential drug target in other protozoan parasites, including apicomplexans (e.g., *Eimeria tenella* and *Toxoplasma gondii*) and kinetoplastid *T. brucei* [133-135]. More recently, three inhibitors (i.e., suramin, agaricic acid, and 5-phosphoarabinonhydroxamic acid) were identified that more selectively inhibited *T. brucei* GPI than the mammalian GPIs [136]. Determining the biochemical properties of CpGPI would increase our knowledge of the

glycolytic pathway in *C. parvum*, and also provide the basis for potentially exploring this enzyme as a new drug target in the parasite.

In the present study, 1,200 existing drugs were screened in the first attempt to identify new CpGPI inhibitors. It was discovered that ebselen could irreversibly inhibit CpGPI (IC_{50} = 8.33 μM), but not HsGPI (at concentrations up to 400 μM). It was also found that ebselen could inhibit the growth of *C. parvum* in vitro (EC_{50} = 165 μM), albeit at a relatively low in vitro safety interval of 4.2 (**Fig. 4B**).

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one; CAS # 60940-34-3) is a synthetic organoselenium drug with anti-oxidant, anti-inflammatory, anti-atherosclerotic, anti-thrombotic, neuroprotective and cytoprotective properties [137-141] (**Fig. 2A**, inset). It has been studied for the treatment of various diseases including arthritis, stroke and atherosclerosis in diabetic patients [139, 142, 143]. This drug also displayed a number of beneficial effects in experimental animal models [144, 145], albeit with certain levels of cellular toxicity (e.g., induction of DNA damage, apoptosis and necrotic cell death).

The toxicological effects of ebselen have not been thoroughly studied. Based on the Registry of Toxic Effects of Chemical Substances (RTECS) data, the lowest oral dose resulting in a toxic effect (TDLO) in mice and rats was estimated at 5 and 10 mg/kg, respectively, and the oral LD50 in mice was determined at 6,810 mg/kg. In humans, a number of therapeutic doses have been reported. For example, ebselen was safe at an oral dose of 600 mg twice daily (eq. ~20 mg/kg for a 60-kg adult) for 4 days, a single dose up to 1,600 mg (~26.7 mg/kg), or 150 mg twice daily (~5 mg/kg) for two weeks [146-148]. Although it is questionable as to whether ebselen could be explored for treating cryptosporidiosis, this study has nonetheless expanded the drug profile of ebselen by discovering its selective anti-CpGPI activity.

Ebselen is known to target several different enzymes, including those from some protozoan parasites (e.g., hexokinase in *Trypanosoma brucei and Plasmodium falciparum*), mainly by chemical modification of the protein SH-groups forming a stable selenosulphide complex [131, 132, 149-154]. Therefore, it is likely that the in vitro anti-cryptosporidial efficacy of ebselen might be attributed to its effects on both CpGPI and other parasite enzymes. Additionally, the safety margin of ebselen observed in this study is small (i.e., in vitro safety interval of 4.2). Although ebselen is useful in studying the inhibition of CpGPI enzyme activity, further proof is needed to chemically and/or genetically validate CpGPI as a drug target.

Collectively, the discovery that ebselen is a selective CpGPI inhibitor provides an opportunity to study the properties of the CpGPI enzyme and its enzyme-inhibitor interactions. This study also provides a basis for the further screening of large compound libraries to identify CpGPI inhibitors for potential development as anti-cryptosporidial therapeutics. CpGPI is a "plant-like" enzyme highly divergent from those in mammals [13]. There is a good opportunity to identify more selective and more potent CpGPI inhibitors for humans and other mammals with greater safety intervals.

3 ACTION OF 2-DEOXY-D-GLUCOSE ON *CRYPTOSPORIDIUM PARVUM* AND DISCOVERY OF ACTIVITIES AGAINST THE PARASITE HEXOKINASE FROM EXISTING DRUGS

3.1 Overview

Human cryptosporidiosis is mainly caused by the infection of Cryptosporidium parvum (zoonotic) and C. hominis (virtually human-specific), causing water-borne outbreaks. It is one of the top 4 diarrheal pathogens in children in developing countries. Its infection is severe or life threatening in immunocompromised individuals. However, the treatment options are limited with only, yet not fully effective, nitazoxanide (NTZ) the FDA-approved drug for use in immunocompetent individuals. Enzymes in the parasite glycolytic pathway are high potential drug targets since this opportunistic pathogen lacks the Krebs (TCA) cycle and cytochrome based-respiration, so it relies mainly, if not only, on glycolysis to produce ATP. Our previous study revealed 2-deoxy-D-glucose can inhibit the C. parvum hexokinase, one of key enzyme in glycolytic pathway, and C. parvum growth in vitro, but the action and fate of 2DG in *C. parvum* was not fully investigated. This study demonstrated that the anti-cryptosporidial activity of 2DG was mainly attributed to the action of 2DG on CpHK, rather than the action of 2DG6P on CpGPI. We also performed a high-throughput screening of 1200 existing drugs using spectrophotometry-based assays for potential CpHK inhibitors. Four drugs (Hexachlorphene, thimerosal, alexidine dihydrochloride and ebselen) are identified as CpHK inhibitors with anti-cryptospordial activities at micromolar levels (EC50: 0.53, 1.77, 8.1 and 165 μM respectively) at concentrations nontoxic to the host cells. The four hits have provided us new anti-CpHK

reagents with diversified chemical structures. Our data on 2DG action supports CpHK can serve as drug target and the four existing drugs can serve as new reagents for studying the enzyme properties.

Cryptosporidium parvum is zoonotic protozoan parasite causing mild to severe watery diarrhea in humans and some other mammals [49]. Cryptosporidial infection can be prolonged and deadly in immunocompromised patients. It is also one of the four pathogens responsible for the most cases of moderate to severe diarrhea (0 – 5 years old) and increased risk of death in children (1 – 2 years old) in low-income countries in sub-Saharan Africa and south Asia [51, 155]. However, fully effective drugs are yet unavailable to treat cryptosporidiosis in humans and animals. In fact, nitazoxanide (NTZ) is the only drug approved by Food and Drug Administration (FDA) in the United States to treat cryptosporidial infection in human patients with a healthy immune system [49, 116, 156, 157].

Unlike other apicomplexans, *Cryptosporidium* has a compact genome that encodes a highly streamlined metabolism. In energy metabolism, *C. parvum* lacks Krebs cycle and cytochrome-based respiration, but relies on glycolysis to synthesize ATP [13, 102, 158]. Its glycolytic pathway may start with a number of mono-, di-, and poly-saccharides (e.g., glucose, mannose, sucrose, amylose and amylopectin), and exits with lactate, ethanol or acetate as the organic end products or provides substrates for the synthesis of trehalose and N-glycans, the elongation of very long chain fatty acids, and the formation of complex lipids. Several unique glycolytic and fermentative enzymes are present in *Cryptosporidium*, such as the hexokinase (HK) more closely related to those from prokaryotes than to those from fungi, animals and plants, two pyrophosphate-dependent phosphofructokinases (PPi-PFKs) that differ from the ATP-dependent PFK in humans and animals, a bifunctional pyruvate:NADP$^+$ oxidoreductase (PNO), and a

bacterial-type lactate dehydrogenase (LDH) [103, 106]. Therefore, the glycolytic and fermentative enzymes are an attractive subject of research not only for the understanding of the parasite metabolism and evolution, but also for the exploration as novel drug targets in the parasite.

Cryptosporidium possesses a single hexokinase [EC: 2.7.1.1] that catalyzes the first committed reaction in the glycolysis of glucose and other hexoses that may be obtained from the host through sugar transporters or by the degradation of amylopectin. We have previously characterized the *C. parvum* hexokinase (CpHK) that showed a number of unique molecular and biochemical features [104]. We also observed that 2-deoxy-D-glucose (2DG), a glucose analog and classic HK inhibitor, was able to inhibit both the enzyme activity of CpHK and the growth of *C. parvum* in vitro. The inhibition of the parasite growth was more effective in the absence or lower concentrations of glucose and could rescued by adding higher levels of glucose, confirming that "*C. parvum* relies heavily on glucose as a carbon source and 2DG likely acted on the glycolytic pathway in the parasite"[104].

However, the action and fate of 2DG in *C. parvum* was not fully investigated. It is known that 2DG can be phosphorylated by human and animal HK to form 2DG-6-phosphate (2DG6P) that cannot be further metabolized. However, it was unclear whether 2DG could also be phosphorylated by CpHK. The anti-cryptosporidial activity of 2DG could be attributed mostly to the action of 2DG on CpHK, but the action of 2DG on the parasite glucose-6P isomerase (CpGPI) catalyzing the second reaction and/or the action of 2DG6P on CpHK or CpGPI might potentially contribute to the observed anti-parasitic activity as well (**Figure 11**).

The present study aimed to address the questions by determining the inhibitory effects of

2DG and 2DG6P on CpHK and CpGPI, and comparing the anti-cryptosporidial activity and cytotoxicity between 2DG and 2DG6P, which would help us to better understand the action of 2DG in the parasite. Additionally, although 2DG can be employed to probe the effects of inhibition of HK, the chance for being developed into anti-cryptosporidial drug is low due to the little selectivity between CpHK and host HKs and its micromolar level of in vitro anti-cryptosporidial activity (i.e., EC_{50} = 0.54 mM) [104]. Therefore, we also initiated a drug screening campaign aimed to discover more selective and more efficacious anti-CpHK inhibitors. As a first step of the campaign, we screened 1200 existing drugs for potential anti-CpHK activities, and identified 4 existing drugs with not only lower micromolar level anti-CpHK enzyme, but also lower micromolar anti-cryptosporidial growth activities as described below. While the screening of larger compound libraries is ongoing for discovering more selective and efficacious anti-CpHK inhibitors, the identification of the four hits from existing drugs has provided us new anti-CpHK reagents with diversified chemical structures.

3.2 Materials and methods

3.2.1 Chemicals and other reagents

Chemicals including substrates and inhibitors at analytical grade or higher purity were purchased from Sigma-Aldrich (St. Louis, MO) or as specified. The compound library containing 1200 existing drugs was purchased from Prestwick Chemical (San Diego, CA) (http://prestwickchemical.com). Molecular kits and reagents were purchased from various resources as specified.

Oocysts of *C. parvum* (Iowa-1 strain) were purchased from Brunch Grass Farm (Deary, ID) and only those less than 3 months old were used in the in vitro drug efficacy assay. Oocysts were

cleaned by a treatment of 10% Clorox in water for 10 min on ice, followed by extensive washes to completely remove the chlorine and suspension in PBS that were used immediately or stored at 4 °C before use.

3.2.2 Preparation of recombinant proteins

The CpHK gene (gene ID: cgd6_3800; GenBank: XM_627719) was previously cloned into an MBPHT-mCherry2 vector for the expression of CpHK as a recombinant protein fused with maltose-binding protein (MBP)-His-mCherry tags (Yu et al. 2014). The CpGPI gene (Gene ID: cgd2_3200; GenBank: XP_626511) was previously cloned into an pMAL-c2E-TEV-His vector for the expression of CpGPI as an MBP-fusion protein (Eltahan et al. 2018). The expression of recombinant CpHK and CpGPI proteins in Escherichia coli and the amylose resin-based affinity purification of recombinant proteins followed standard protocols as described (Guo and Zhu 2012).

For determining the selectivity of inhibitors, we also expressed the human (Homo sapiens) HK I (HsHK1) as an MBP-fusion protein. HsHK1 was selected from the four HKs presented in mammals because it is the house-keeping enzyme found in all mammalian tissues. For cloning, a plasmid containing the cDNA of the open reading frame (ORF) of HsHK1 gene (GenBank: NM_000188.2) was purchased from GeneCopoeia (Rockville, MD). The plasmid was used as a template for amplifying the 2754-bp HsHK1 ORF by a high-fidelity PCR using a HiFi PCR Kit (KAPA Biosystems, Wilmington, Massachusetts) and subcloned into pMAL-c2E-TEV-His vector at the EcoRI and HindIII restriction sites using NEB assembly kits (New England BioLabs Inc.). Primers used for HsHK assembly are HsHK_assemlby_F (att ttc agg gca tgg tac cgA TGA TCG CCG CGC AGC TC′) and HsHK_assembly_R (tgg tgg tga tga tgg tgg tgg tgG

CTG CTT GCC TCT GTG C). Clones were screened by PCR for the presence of inserts in plasmids, and a selected number of plasmids containing correctly oriented HsHK1 inserts were sequenced to confirm the accuracy of the inserts. The expression of recombinant MBP-HsHK1 protein was carried out in a Rosetta 2 strain of E. coli cells. The induction of expression and purification of MBP-HsHK1 protein followed standard protocols (Guo and Zhu 2012, Eltahan et al. 2018, Yu et al. 2014).

The MBP-tag was similarly expressed and purified from the blank pMAL-c2E-TEV-His plasmid for use as a negative control in biochemical analysis of recombinant proteins. The quality and quantity of MBP-tag and recombinant proteins including MBP-CpHK, CpGPI and MBP-HsHK1 were evaluated by SDS-PAGE analysis and Bradford protein assay using bovine serum albumin (BSA) as the standard.

3.2.3 Biochemical analysis

HK assays. The CpHK and HsHK1 enzyme activities were evaluated by two assays as previously described (Yu et al. 2014). The first one was an HK/glucose-6-phosphate dehydrogenase (G6PDH)-coupled assay that detected the product glucose-6P. Briefly, the assay was performed in 200 µL reactions containing D-glucose (2 mM or as specified), ATP (2.5 mM or as specified), NAD+ (0.3 mM), G6PDH (2 U), MgCl2 (5 mM) in Tris.HCl buffer (50 mM, pH 7.5), and MBP-CpHK or HsHK1 (500 ng) in the absence or the presence of an inhibitor at specified concentrations. In this assay, glucose was phosphorylated to form glucose-6P by HK, and then glucose-6P was converted into glucono-1′5-lactone-6P by G6PDH. The consumption of NAD+ was monitored spectrophotometrically at a 340 nm every minute for up to 30 min using Multiscan Spectrum spectrophotometer (Thermo Scientific, Waltham, MA).

When an inhibitor was tested by the HK/G6PDH-coupled assay, the observed inhibitory effect might possibly be attributed to the action of the inhibitor on G6PDH. This possibility was tested by G6PDH assay, which was performed similarly to the G6PDH-coupled assay in the absence of HK and glucose, but using glucose-6P (0.2 mM) as the substrate. A lower concentration of G6PDH (0.04 U) was also used to avoid the potential masking of the inhibitory effect on G6PDH (Yu et al. 2014).

The second one was a pyruvate kinase/lactate dehydrogenase (PK/LDH)-coupled assay. This assay detected the formation of ADP, thus allowing the evaluation of HK activity towards various substrates in addition to glucose, such 2DG in this study. It was performed in 200 µL reactions containing D-glucose (5 mM) or other substrates (e.g., 2DG) at specified concentrations, ATP (2.5 mM), phosphoenolpyruvate (PEP; 1 mM), NADH (0.15 mM), PK (5 U), rabbit muscle LDH (10 U), and MBP-CpHK, MBP-HsHK1 or MBP (500 ng). In this assay, ADP was converted back to ATP to produce pyruvate from PEP by PK. Pyruvate was then converted to lactate and the consumption of NADH was monitored spectrophotometrically at a 340 nm every minute for up to 30 min. G6PDH, PK and LDH used in these assays were purchases from Sigma Aldrich (St. Louis, MO).

GPI assay. The potential inhibitory effect of 2DG and 2DG6P on CpGPI was evaluated by a GPI/G6PDH-coupled assay as previously described (Eltahan et al. 2018). It was performed similarly to the HK/G6PDH-coupled assay, but in a reverse reaction direction that converted fructose-6P into glucose-6P by CpGPI. Glucose-6P was then converted into glucono-1,5-lactone-6-phosphate by G6PDH using NAD+ as a cofactor. A typical assay was performed using 200 µL

reactions containing D-fructose-6P (2 mM), NAD+ (0.2 mM), G6PDH (2 U), MgCl2 (5 mM) in Tris.HCl buffer (50 mM, pH8.5), and MBP-CpGPI or MBP (50 ng).

All assays were performed at least twice in triplicated reactions at room temperature (23 °C) except for the reactions involving HsHK1 enzyme that was assayed at 37 °C due to its low activity at room temperature. Enzyme kinetics were analyzed using GraphPad Prism version 5.0f or higher (http://www.graphpad.com). MBP-tag was used as the negative control in all assays for background subtraction.

3.2.4 High-throughput (HTS) of screening of existing drugs

The Prestwick chemical library containing 1200 existing drugs were screened in 96-well plates for potential anti-CpHK activities using the HK/G6PDH-coupled assay as described above. All drugs were screened at of 20 µM in primary screening. Each reaction was performed at least twice independently at room temperature. Negative controls using MBP-tag (2 wells/plate) and positive controls using 2DG (5 mM; 2 wells/plate) were included in each plate. Top hits were then evaluated by G6PDH assay to exclude those showing significant anti-G6PDH activities from further analysis as described above. Hits with confirmed anti-CpHK activities were subjected to further analysis to determine their inhibitory kinetic parameters on CpHK in comparison with those of HsHK1. Because of the low activity of HsHK1 at room temperature, detailed comparison on the effects of inhibitors on CpHK and HsHK1 were performed at 37 °C. All reactions contained 1% DMSO that was used to increase the solubility of compounds and performed at least twice in triplicates.

3.2.5 *Drug efficacy against the parasite growth in vitro*

The efficacies of 2DG, 2DG6P and hits identified from existing drugs were evaluated against the growth of C. parvum in vitro using a qRT-PCR assay as previously described (Cai et al. 2005, Zhang and Zhu 2015, Guo et al. 2018). Briefly, HCT-8 cells (ATCC # CCL-225) were cultured overnight in 96-well plates in RPMI-1640 medium containing 10% fetal bovine serum (FBS) at 37 °C under 5% CO2 atmosphere until they reached to ~80% confluence. Oocysts were then added to the host cells monolayers (oocyst:host cell ratio = 1:2; i.e., ~2×104 oocysts with ~4×104 host cells/well) and incubated for 3 h at 37 °C, followed by a medium exchange to remove uninfected parasites and the addition of compounds at specified concentrations. Infected cells were incubated for an additional 41 h (total 44 h infection time). Cell lysates were prepared, diluted, and used directly to evaluate the parasite loads by qRT-PCR in 384-well plates as described (Zhang and Zhu 2015). Paromomycin (0.14 mM) and 2DG (0.5 mM) were used as positive controls and diluent (1% DMSO) is used as negative control in the assay. Cytotoxicity of drugs was evaluated by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In addition, the relative levels of 18S rRNA in uninfected HCT-8 cells was also evaluated for cytotoxicity. All assays were performed at least twice independently in triplicates. Data was analyzed with Microsoft Excel and GraphPad Prism version 5.0f or higher.

3.3 Results

3.3.1 *The anti-cryptosporidial activity of 2-deoxy-D-glucose (2DG) was mainly attributed to its action on the parasite hexokinase (CpHK)*

We have previously shown that 2DG could inhibit CpHK enzyme activity (IC_{50} = 5.75 mM; K_i = 0.34 mM) and the growth of *C. parvum* (EC_{50} = 0.54 mM) at concentrations non-toxic to

host cells [104]. To fully understand the action of 2DG in the parasite, we wanted to determine whether CpHK could catalyze 2DG to form 2DG6P and whether 2DG6P could act on the downstream enzyme CpGPI to contribute to the anti-cryptosporidial activity.

We first confirmed that, similar to HsHK1, CpHK could utilize 2DG as a substrate to form 2DG6P (K_m = 0.204 mM), but with ~4.5-fold lower affinity in comparison to its activity towards glucose (K_m = 0.046 mM) (Table 2). The activities of CpHK on glucose and 2DG were comparable to HsHK1 (i.e., K_m = 0.265 mM on 2DG and K_m = 0.027 mM on glucose) (Table 2). The data also confirmed that the inhibition of 2DG on CpHK and HsHK1 was due to its direct competition with glucose and its action as a slow substrate of HKs.

However, 2DG6P was infective on both CpHK and CpGPI at concentrations up to 28 mM (the maximal concentration achievable in the assay); while 2DG and its analog fluorodeoxyglucose (FDG) inhibited CpHK activity at low millimolar levels (IC_{50} = 5.75 mM and 3.7 mM, respectively), although 2DG and FDG were ineffective on CpGPI at concentrations up to 50 mM and 150 mM respectively (*Table 3*). For comparison, 2DG6P was also ineffective on HsHK1, whereas 2DG was effective on HsHK1 (IC_{50} = 5.75 mM) (*Table 3*).

These observations were correlated well with the in vitro efficacy data on the parasite, in which 2DG and FDG inhibited the parasite growth at lower millimolar levels (EC_{50} = 0.54 mM and 0.30 mM, respectively), whereas 2DG6P could inhibit the parasite growth at concentrations of more than 33-fold higher than 2DG (i.e., EC_{50} = 18.0 mM) (*Table 3*). In contrast, host cells were much less sensitive to the inhibition of 2DG (TC_{50} = 36.0 mM; in vitro safety interval (SI) = 66.7), 2DG6P (TC_{50} >72.0 mM; SI >4.0) and FDG (TC_{50} >72 mM; SI >240) (Table 4).

Collectively, our data indicated that the anti-cryptosporidial activity of 2DG was mainly

attributed to the action of 2DG on CpHK, rather than the action of 2DG6P on CpGPI (**Figure 11**).

3.3.2 Four existing drugs were discovered to possess anti-CpHK activities

As a first step to discover new CpHK inhibitors, we screened 1200 existing drugs in the Prestwick Chemical Library using an HK/G6PDH-coupled assay. The assay was validated by the NIH-recommended intra-plate, inter-plate, and inter-day uniformity tests. Signal window (*SW*) and Z′ factor for each plate were calculated according to the Assay Guidance Manual as follows [159]

$$SW = \frac{(AVG_{max} - 3SD_{max}/\sqrt{n}) - (AVG_{min} + 3SD_{min}/\sqrt{n})}{SD_{max}/\sqrt{n}}$$

SW=(AVGmax-3SDmax⁄n)-(AVGmin+3SDmin⁄n)SDmax⁄n

$$Z' = \frac{(AVG_{max} - 3SD_{max}/\sqrt{n}) - (AVG_{min} + 3SD_{min}/\sqrt{n})}{AVG_{max} - AVG_{min}}$$

The SW and Z′ values in the nine plates were ranging from 7.1 to 37.59 and 0.70 to 0.92, respectively which are higher than the recommended acceptance criterions (i.e., SW ≥ 2 and Z′ ≥ 0.4). Our primary screening identified 21 compounds with ≥ 50% inhibition of enzyme activity at 20 µM (**Figure 7**C). The 21 compounds were subjected to a G6PDH assay using lower concentrations of G6PDH (0.04 U) in the absence of CpHK and glucose, by which twelve compounds were confirmed to be specific. Among them, 8 hits were excluded for further assays due to cytotoxicity or insolubility at applicable concentrations. The remaining 4 specific CpHK inhibitors are thimerosal, alexidine dihydrochloride (alexidine•2HCl), hexachlorophene and ebselen (**Figure 7**B).

Among the 4 hits, ebselen was previously found to also inhibit CpGPI [160]. However, other studies had showed that ebselen could act on as a reversible inhibitor of *Plasmodium*

falciparum HK (PfHK) or an irreversible inhibitor for *Trypanosoma brucei* HK (TbHK) [132, 152], so that it was not a surprise to also see its inhibition on CpHK. We observed that, similar to its action on CpGPI and TbHK, ebselen was an irreversible inhibitor of CpHK, as the inhibition could not be reversed by up to 50-fold dilution of the inhibitor (i.e., from 100 to 2 μM) [132, 160](**Figure 10A**). Additionally, the reducing agent DTT (dithiothreitol) at 100 mM could prevent the inhibition when incubated with the enzyme prior to the reaction and partially reversed the inhibition when added after incubation of ebselen with enzyme (**Figure 10B**). These observations suggest that ebselen might covalently bind to CpHK, likely via the modification of Cys residues of CpHK, while the direct contact between DTT and ebselen could interfere the enzyme-inhibitor association at certain levels.

All four hits were further analyzed to determine their inhibitory kinetics and IC_{50} values on CpHK and HsHK. Ebselen (IC_{50} = 0.241 μM) and alexidine•2HCl (IC_{50} = 22.1 μM) acted as noncompetitive inhibitor of CpHK, while the action of thimerosal (IC_{50} = 2.3 μM) and hexachlorophene (IC_{50} = 8.8 μM) on CpHK fit with the mixed model of inhibition. They also showed very similar IC_{50} values on HsHK1 (**Figure 8**), suggesting no selectivity of the four compounds between CpHK and HsHK1.

Despite of the lack of selectivity between the parasite and host HK enzymes, the four compounds affect the growth of *C. parvum* in vitro more that of the host cells. The in vitro safety intervals (SI; SI = TC_{50}/EC_{50}) ranged from ~4 for alexidine•2HCl and ebselen to 10.5 for thimerosal and 188.7 for hexachlorophene, indicating that the parasite was much more sensitive than host cells to the inhibition by the four compounds, particularly by hexachlorophene (Table 5).

3.4 Discussion

In the present study, we first confirmed that 2DG could be phosphorylated by CpHK to form 2DG6P, but the phosphorylated form of 2DG was ineffective on CpGPI, CpHK and HsHK1 at concentrations comparable to or higher than the effective concentrations of 2DG (**Table 2**). 2DG6P was much less effective than 2DG on the parasite grown in vitro and host cells (Table 4), which validated that the anti-cryptosporidial action of 2DG was attributed to its action on CpHK (**Figure 11**). These observations further support the notion that CpHK is a valid drug target in the parasite.

As discussed earlier, the chance to develop 2DG into an anti-cryptosporidial therapeutic might be low, albeit further investigation [104]. However, 2DG can serve as a useful reagent to study the function of CpHK in the parasite. Because 2DG can be converted to 2DG6P, leading to the loss of inhibitory effect on the parasite, it would be highly appealing to design/identify 2DG analogs that cannot be phosphorylated by CpHK to extend its action on CpHK and increase its efficacy on the parasite. This notion is partly supported by the data on FDG that displayed slightly better anti-CpHK activity (IC_{50} = 3.7 mM on CpHK, vs. 5.75 mM by 2DG) (*Table 3*), and slightly improved in vitro anti-*C. parvum* efficacy (EC_{50} = 0.3 mM, vs. 0.54 mM by 2DG) and safety interval (>240-fold, vs. 66.7 for 2DG) (Table 4).

Secondly, we initialized a drug screening campaign for identifying new anti-CpHK compounds by first screening 1200 existing drugs. Among the four anti-CpHK hits, only ebselen can be orally administrated for medical use [141, 143, 146, 161, 162] Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one; CAS # 60940-34-3) is a synthetic organoselenium known to have multiple targets enzymes in many species including HKs from several protozoan parasites [131, 132, 150-152, 163, 164]. It was also recently identified as the only inhibitor of CpGPI among

existing drug [160]. It is likely that ebselen might also affect other Cys-containing enzymes in the parasite. Although the efficacy of ebselen at micromolar levels was not impressive (EC_{50} = 0.165 mM), there could be a chance to investigate its analogs to identify more efficacious and selective inhibitors.

The other three hits were antiseptic biguanide (alexidine•2HCl), organochlorine (hexochlorophene) and organomercury (thimerosal) compounds [165, 166]. These compounds were also reported to possess activities against some parasites, such as the anti-*Acanthamoeba* activity of alexidine [167], anti-*Plasmodium* and anti-trypanosome activities of thimerosal [168, 169], and anti-nematode and anti-protozoan activities of hexochlorophene [170-173]. On the other hand, these compounds are only labeled for use as vaccine preservative or disinfectants, rather than for oral medication, for which they are unlikely to be developed into anti-cryptosporidial therapeutics.

In summary, our study on the action of 2DG further supported that CpHK could serve as a drug target in the parasite, and the drug screening identified four existing drugs as CpHK inhibitors that could be used as new reagents for studying the CpHK enzyme properties. We are currently screening large compound libraries including the TimTec Diversity Screening Set of 10,000 compounds (http://www.timtec.net) aimed to identify more efficacious and selective anti-CpHK leads for developing anti-cryptosporidial therapeutics, and will report our findings in the near future.

Table 2 CpHK and HsHK1 enzyme parameters towards D-glucose and 2-deoxy-D-glucose (2DG) as determined by HK/G6PDH-coupled assay

Substrate	CpHK		HsHK1	
	K_m (mM)	V_{max} (U) [1]	K_m (mM)	V_{max} (U) [1]
D-glucose	0.046	155.2	0.027	53.3
2DG	0.204	111.5	0.265	68.4
Ratio (2DG/glucose)	4.43	0.72	9.81	1.28

[1] U = nmol/μg/min.

Table 3 Effects of 2-deoxy-D-glucose (2DG), 2-deoxy-D-glucose-6-phosphate (2DG6P) and fluorodeoxyglucose (FDG) on CpHK, HsHK1 and CpGPI enzyme activities

Inhibitor	Effects of compounds on enzymes		
	CpGPI	**CpHK**	**HsHK1**
2DG	Ineffective at ≤50 mM	IC_{50} = 5.75 mM	IC_{50} = 10.1 mM
2DG6P	Ineffective at ≤28 mM	Ineffective at ≤28 mM	Ineffective at ≤28 mM
FDG	Ineffective at ≤150 mM	IC_{50} = 3.7 mM	IC_{50} = 15.2 mM

Table 4 In vitro efficacy of 2-deoxy-D-glucose (2DG), 2-deoxy-D-glucose-6-phosphate (2DG6P) against the growth of C. parvum (44 h infection assay) and cytotoxicity on the HCT-8 cells (44 h treatment)

Inhibitor	In vitro efficacy (EC_{50}, mM)	Cytotoxicity (TC_{50}, mM)	Safety Interval (TC_{50}/EC_{50})
2DG	0.54	36.0	66.7
2DG6P	18.0	>72.0	>4.0
2FDG	0.30	>72.0	>240

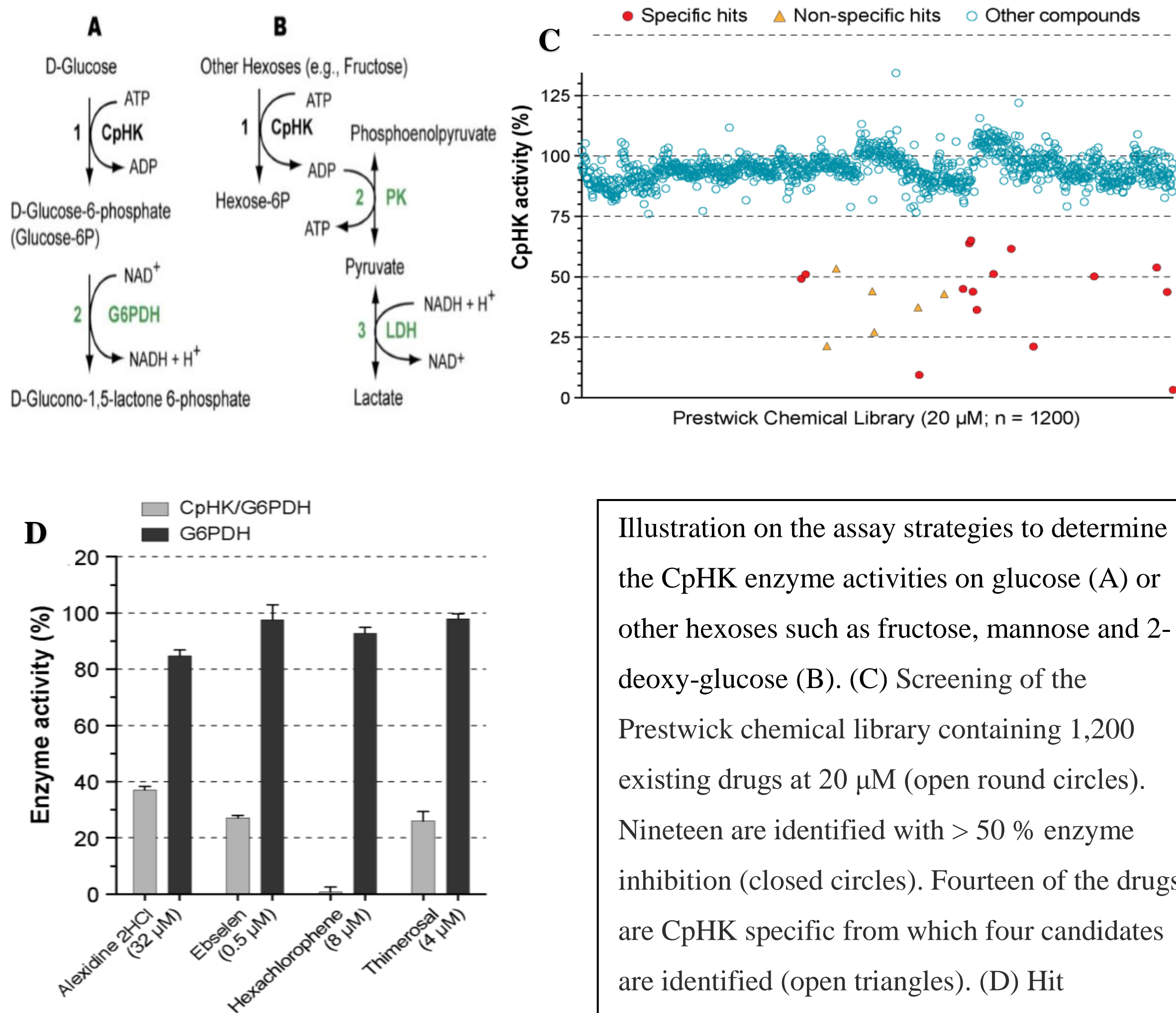


Illustration on the assay strategies to determine the CpHK enzyme activities on glucose (A) or other hexoses such as fructose, mannose and 2-deoxy-glucose (B). (C) Screening of the Prestwick chemical library containing 1,200 existing drugs at 20 μM (open round circles). Nineteen are identified with > 50 % enzyme inhibition (closed circles). Fourteen of the drugs are CpHK specific from which four candidates are identified (open triangles). (D) Hit validation assay showing four authentic hits because they were ineffective on G6PDH used in the CpHK/G6PDH-coupled assay, with no inhibition on G6PDH. Bars represent standard errors of the mean (SEMs) derived from at least three replicated reactions.

Figure 7 Identification of four drugs as inhibitors of CpHK from existing drugs of Prestwick chemical library

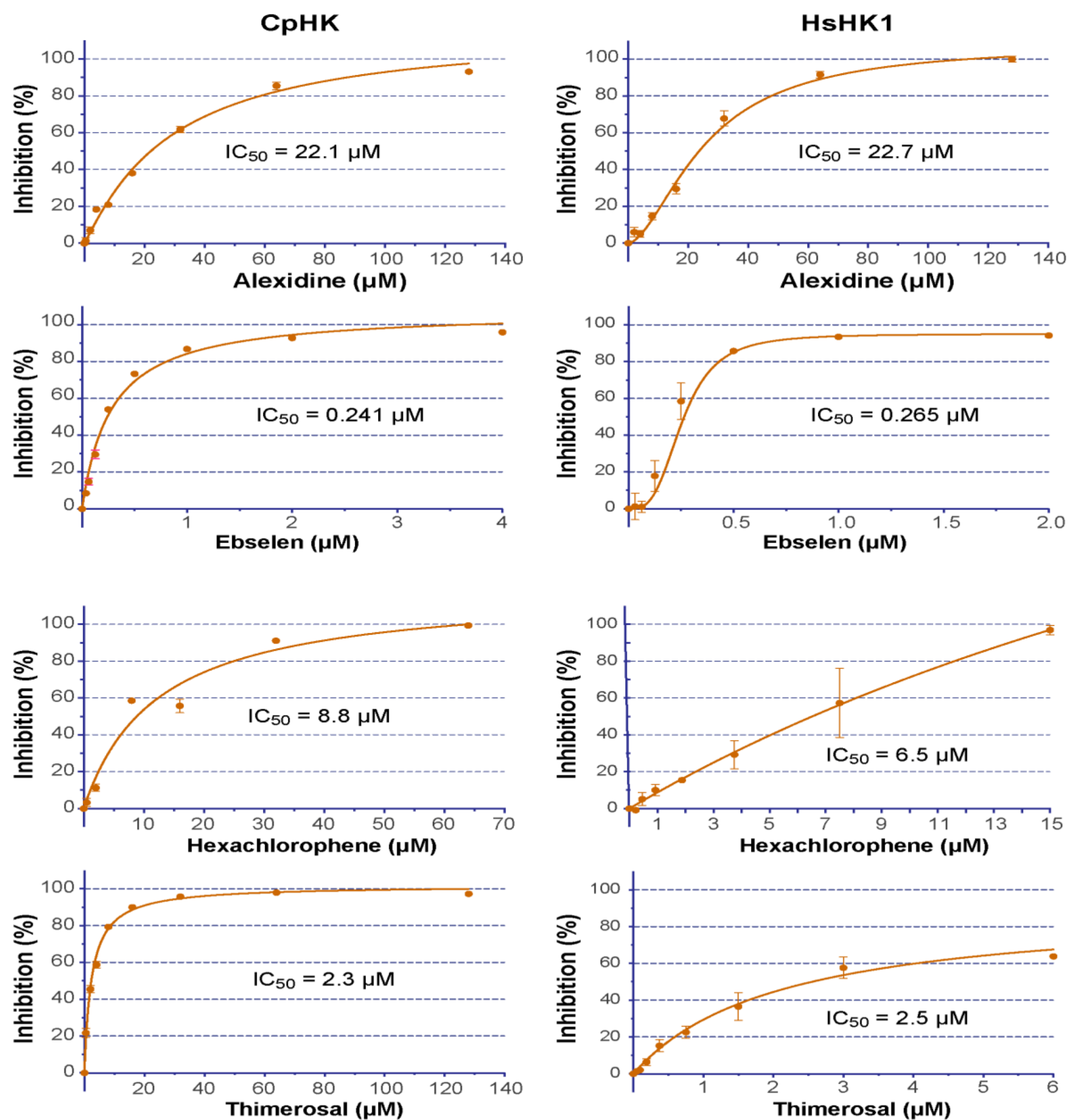
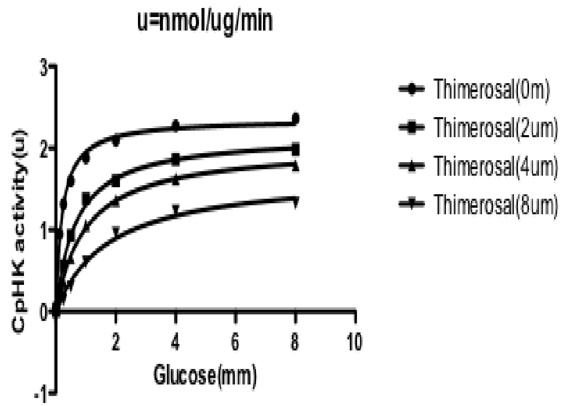
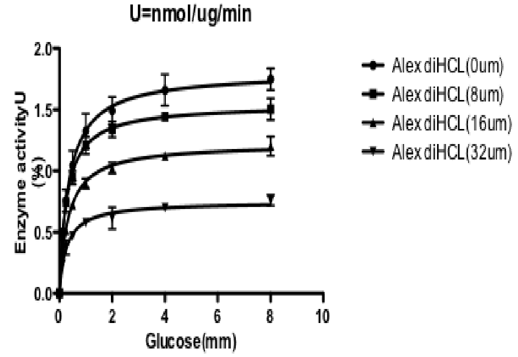


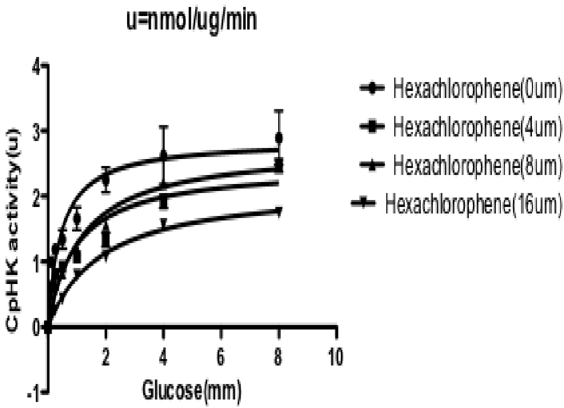
Figure 8 Dose-dependent inhibition of drug candidates on CpHK (left panel) and HsHK1(Right panel) for determining the IC_{50} values. Bars represent standard errors of the mean (SEMs) derived from at least three replicated reactions.

A

B







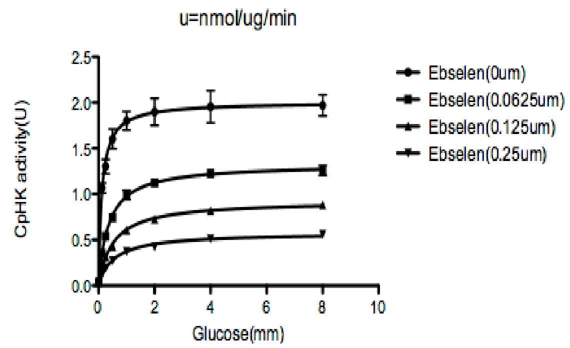


Figure 9 Allosteric kinetics of the drug kinetics with CpHK (A) Apparent kinetics of thimerosal and hexachlorophene fitted with the competitive inhibition model; (B) Alexidine dihydrochloride and ebselen fitted with the non-competitive inhibition model. Bars represent standard errors of the mean (SEMs) derived from at least three replicated reactions.

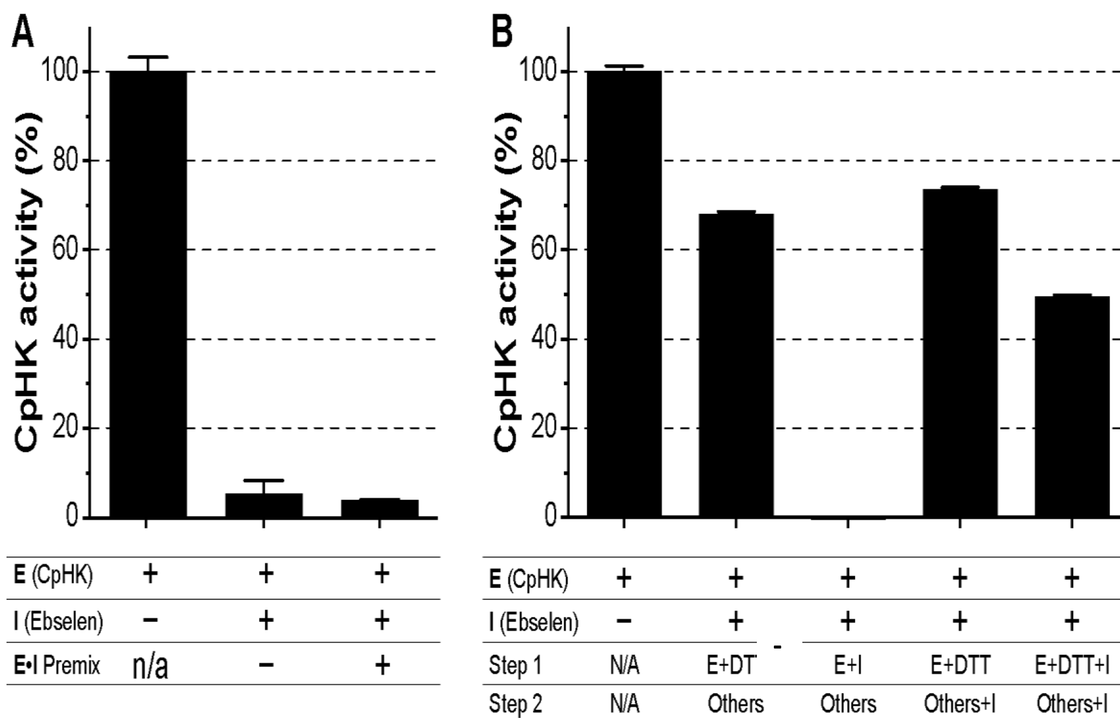


Figure 10 Effects of dilution and DTT on the inhibition of ebselen (Ebs) on CpHK

(A) In the inhibitor dilution assay, pre-incubation of ebselen (100 μM) with CpHK on ice for 10 min followed by 50-fold dilution with the addition of other reaction components had no effect on the inhibition of ebselen on CpHK (B) In the DTT assay, pre-incubation of DTT (100 mM) and CpHK only slightly affected the enzyme activity (lane 2), but blocked the inhibition of ebselen on CpHK (lane 4). DTT partially reversed ebselen inhibition without its prior incubation with CpHK (lane 5). Bars represent standard errors of the mean (SEMs) derived from at least three replicate d reactions.

Table 5 Inhibition of the CpHK and HsHK1 enzyme activity by the top four hits identified from 1200 existing drugs and their efficacies against the growth of C. parvum in vitro

Compound	Inhibition on CpHK (IC_{50}, µM)	Inhibition on HsHK1 (IC_{50}, µM)	Selectivity on enzyme	Efficacy on *C. parvum* (EC_{50}, µM)	Cytotoxicity (TC_{50}, µM)	Safety interval (SI)	Compound description
Alexidine•2HCl	22.1	22.7	1.03	8.1	32.6	4.02	Biguanide compound; Antibacterial, detergent, oral disinfectant, apoptosis promotic agent
Ebselen	0.241	0.265	1.10	165*	700*	4.24	Organoselenium compound; Anti-inflammatory, anti-thrombotic, anti-atherosclerotic, anti-oxidant
Hexachlorophene	8.8	6.5	0.74	0.53	100	188.7	Organochlorine compound; Antiseptic, bacteriostatic, anti-helminthic
Thimerosal	2.3	0.35	1.52	1.77	18.5	10.5	Organomercury compound; Antiseptic, antifungal, vaccine preservative

* Data adapted from a previous study (Eltahan et al, 2018).

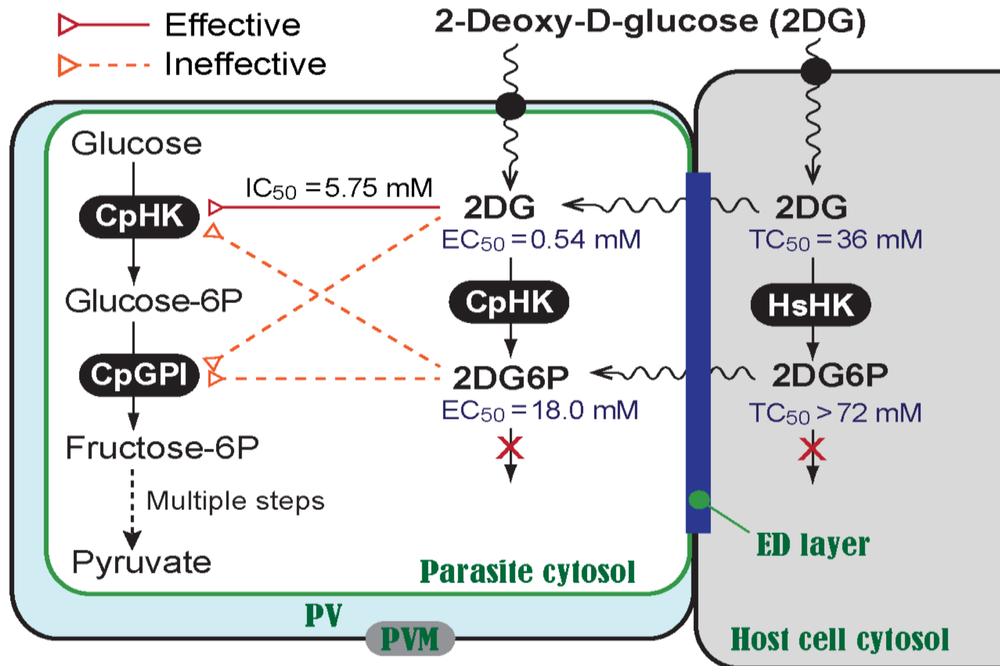


Figure 11 Potential action of 2DG in *C. parvum* cell leading to the inhibition of parasite growth by blocking the glycolysis. 2DG inhibited CpHK and HsHK1 (solid lines) but was ineffective on CpGPI up to 50 mM (dotted lines). 2DG6P was ineffective (dotted lines) against CpGPI, CpHK and HsHK up to 28 mM.

4 SUMMARY AND CONCLUSIONS

Cryptosporidium parvum is a unicellular protozoan that infects wide range of host including humans and other animals. It is one of the top 4 diarrheal pathogens in children in developing countries in which they suffer from growth retardation and also associated with fatalities in toddlers. The preventative measures are not fully effective and the treatment options are limited. Up to date, Nitazoxanide (NTZ) is the only FDA-approved drug for use in immunocompetent patients with no treatment yet for animals. Although, HAART therapy can be provided for treatment of cryptosporidiosis in immunocompromised patients but it is life threatening. Therefore, there is a strong urge to develop new and effective anti-cryptosporidial drugs.

Fortunately, the whole genome of *C. parvum* is sequenced revealing the unique metabolic features of *C. parvum.* It lacks the mitochondria genome and apicoplast, unlike other apicomplexans examined so far. Since, it lacks the TCA (krebs cycle) and cytochrome-based respiration, it relies mainly, if not only, on glycolysis for energy source. It also lacks the denovo synthetic pathways (amino acids, fatty acids and nucleotides) so it lacks various pathways that serves as potential drug targets in other apicomplexans. Therefore, it is worth exploring the glycolytic enzymes as potential drug targets for developing novel therapeutics.

In this study, we characterized the primary biochemical features of the glycolytic enzyme, *C. parvum* glucose-6-phosphate isomerase (CpGPI). By high-throughput screening of 1,200 known drugs using spectrophotometer based- assays, we discovered ebselen, was an inhibitor of CpGPI. Ebselen acted on CpGPI as an allosteric noncompetitive inhibitor. We also identified thimerosal, hexachlorophene, alexidine dihydrochloride and ebselen as CpHK inhibitors. Thimerosal and hexachlorphene acted as competitive inhibitors, while alexidine dihydrochloride and ebselen

were noncompetitive inhibitors of CpHK. The four drugs can also inhibit the growth of *C. parvum* in vitro at concentrations nontoxic to host cells by qRT-PCR. Additionally, ebselen might also target other enzymes in the parasite, leading to the parasite growth reduction. Collectively, the discovery of these drugs as selective CpGPI/CpHK inhibitors provides an opportunity to study the properties of the enzymes and its enzyme-inhibitor interactions. This study also provides a basis for the further screening of large compound libraries to identify new chemical structures as inhibitors for potential development as anti-cryptosporidial therapeutics. Added to this, CpGPI is a “plant-like” and CpHK is a “bacterial-like” enzyme which are highly divergent from those in mammals [13, 104]. There is a good opportunity to identify more selective and more potent enzyme inhibitors for humans and other mammals with greater safety intervals.

Moreover, our previous study revealed 2-deoxy-D-glucose (2DG) can inhibit CpHK and *C. parvum* growth invitro but the potential mechanism of action is not yet clear. This study showed 2DG was at least 30 folds more potent than 2-deoxy-D-glucose-6 phosphate (2DG6P). Together with inability of 2DG6P to inhibit both, CpGPI and CpHK enzymes, our study suggests the inhibitory effect of 2DG on the parasite growth is mostly attributed to its inhibition on CpHK rather than by its metabolizable product, 2DG6P. It may be rewarding to explore 2DG, previously investigated as anti-viral and anti- cancerous agent in clinical trials, as a new therapeutic drug for cryptosporidiosis. Also, this study will open avenues to study other and possibly more potent sugar analogues with anti-cryptosporidial activities. Therefore, although the 2DG and the drug candidates are useful in studying the inhibition of CpGPI and CpHK enzyme activities, further proof is needed to chemically and/or genetically validate both enzymes as drug targets.

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