IJP: Drugs and Drug Resistance 8 (2018) 43–49

Contents lists available at ScienceDirect

IJP: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Discovery of ebselen as an inhibitor of *Cryptosporidium parvum* glucose-6-phosphate isomerase (CpGPI) by high-throughput screening of existing drugs

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ARTICLE INFO

Keywords:
Apicomplexan
Cryptosporidium parvum
Glucose-6-phosphate isomerase (GPI)
Ebselen

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. 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 a selective inhibitor of CpGPI by high-throughput screening of 1200 known drugs. Ebselen acted on CpGPI as an allosteric noncompetitive inhibitor (IC_{50} = 8.33 μM; K_i = 36.33 μM), while complete inhibition of CpGPI activity was not achieved. Ebselen could also inhibit the growth of *C. parvum* in vitro (EC_{50} = 165 μM) at concentrations nontoxic to host cells, albeit with a relatively small in vitro safety window of 4.2 (cytotoxicity TC_{50} on HCT-8 cells = 700 μM). Additionally, ebselen might also target other enzymes in the parasite, leading to the parasite growth reduction. Therefore, 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.

1. Introduction

Cryptosporidium parasites are the causative agents of cryptosporidiosis in humans and animals (Checkley et al., 2015; Chen et al., 2002; Feng and Xiao, 2017). 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 (Collaborators, 2017; Kotloff et al., 2013; Sow et al., 2016). Additionally, *Cryptosporidium* can cause opportunistic infection with prolonged, life-threatening diarrhea in AIDS patients (Checkley et al., 2015; Chen et al., 2002). 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 (Chen et al., 2002; Kelly, 2011; Mead, 2014). 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) (Abrahamsen et al., 2004; Xu et al., 2004). 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 (Zhu et al., 2000). 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 (Abrahamsen et al., 2004; Rider and Zhu, 2010). 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

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https://doi.org/10.1016/j.ijpddr.2018.01.003
Received 26 October 2017; Received in revised form 14 January 2018; Accepted 17 January 2018

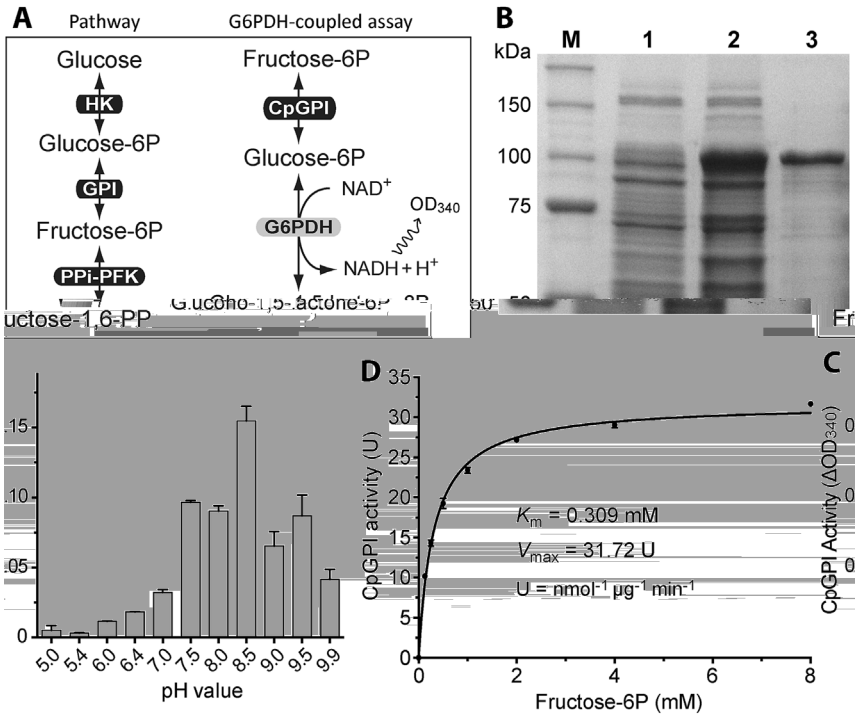

Fig. 1. Enzyme kinetic features of CpGPI.
A) Illustrations of the reaction catalyzed by GPI and the G6PDH-coupled assay for detecting the CpGPI enzyme activity; **B**) SDS-PAGE gel image showing the expression and purification of recombinant CpGPI as an MBP-fusion protein. Lane M, protein markers; Lanes 1 and 2, total proteins from transformed bacteria before and after 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.

polysaccharides (Thompson et al., 2005; Yu et al., 2014). 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. Materials and methods

2.1. Molecular cloning of CpGPI gene and expression of 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 min and then washed 5–8 times in pure water (Arrowood and Sterling, 1987). 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 CC**A TGC CAG AAC TTT ATG AAC 3′) and CpGPI-R-SalI (5′ AT**G TCG AC**A TTC GTC AGG CTC TTT GAA 3′) (Note: bold fonts indicate restriction sites). 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 sequence 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 (Guo and Zhu, 2012). 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 (Guo and Zhu, 2012). 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. 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 1200 known drugs (http://prestwickchemical.com) to identify potential anti-CpGPI activities. Each reaction in the primary screening was performed at least twice