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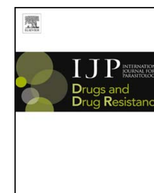

Characterization and validation of *Entamoeba histolytica* pantothenate kinase as a novel anti-amebic drug target

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A B S T R A C T

The Coenzyme A (CoA), as a cofactor involved in > 100 metabolic reactions, is essential to the basic biochemistry of life. Here, we investigated the CoA biosynthetic pathway of *Entamoeba histolytica* (*E. histolytica*), an enteric protozoan parasite responsible for human amebiasis. We identified four key enzymes involved in the CoA pathway: pantothenate kinase (PanK, EC 2.7.1.33), bifunctional phosphopantothenate-cysteine ligase/decarboxylase (PPCS-PPCDC), phosphopantetheine adenylyltransferase (PPAT) and dephospho-CoA kinase (DPCK). Cytosolic enzyme PanK, was selected for further biochemical, genetic, and phylogenetic characterization. Since *E. histolytica* PanK (EhPanK) is physiologically important and sufficiently divergent from its human orthologs, this enzyme represents an attractive target for the development of novel anti-amebic chemotherapies. Epigenetic gene silencing of *PanK* resulted in a significant reduction of PanK activity, intracellular CoA concentrations, and growth retardation *in vitro*, reinforcing the importance of this gene in *E. histolytica*. Furthermore, we screened the Kitasato Natural Products Library for inhibitors of recombinant EhPanK, and identified 14 such compounds. One compound demonstrated moderate inhibition of PanK activity and cell growth at a low concentration, as well as differential toxicity towards *E. histolytica* and human cells.

1. Introduction

Coenzyme A (CoA) is an essential cofactor in all living organisms as an acyl group carrier and carbonyl-activating group involved in more than 100 cellular reactions (Begley et al., 2001); it is estimated to be a cofactor used in 9% of identified enzymatic reactions (Strauss, 2010). CoA biosynthesis is considered to be an essential and universal pathway of the majority of prokaryotes and eukaryotes (Leonardi et al., 2005b). In general, CoA participates in fatty acid metabolism, the tricarboxylic acid cycle and numerous other intermediary metabolic reactions (Abiko, 1975). CoA is synthesized from pantothenate (vitamin B_5), cysteine, and ATP (Jackowski, 1996; Leonardi et al., 2005b). Most of the eukaryotes are unable to synthesize pantothenic acid and thus rely on an external supply.

Entamoeba histolytica is the protozoan agent responsible for human amebiasis, an infectious disease causing dysentery and amebic liver abscesses, responsible for 100,000 deaths annually throughout the world. It represents the third most common parasitic cause of death, after malaria and schistosomiasis (Stanley, 2003; Ali and Nozaki, 2007; Ralston and Petri, 2011). The elaborate pathogenesis of this parasite is well documented (Espinosa-Cantellano and Martínez-Palomo, 2000; Nozaki and Bhattacharya, 2015). Metronidazole has, for decades, been the most effective drug in the treatment of amebiasis despite its known side effects and low efficacy against asymptomatic cyst carriers. Moreover, resistance, virulence and host immune response to metronidazole treatment in amebiasis have been reported in some countries (Griffin, 1973; Pittman and Pittmann, 1974; Johnson, 1993; Koch et al., 1997; Ali and Nozaki, 2007). The molecular target of metronidazole has been well described as a key metabolic enzyme, pyruvate: ferredoxin oxidoreductase, which is involved in acetyl CoA

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production from pyruvate as part of central energy metabolism. Identification and characterization of novel drug targets unique to *E. histolytica* are therefore needed to design better therapeutics against amebiasis.

Here we investigate the CoA biosynthetic pathway of *E. histoltyica*. We identified four enzymes, including one bifunctional enzyme, involved in this pathway. We further characterized one of these enzymes, pantothenate kinase (PanK, EC 2.7.1.33), with biochemical and reverse genetic approaches. Moreover, we identified *E. histolytica* PanK inhibitors by screening the Kitasato Natural Products Library against EhPanK recombinant enzyme. Taken together, we demonstrate that the CoA biosynthetic pathway, in general, and PanK, specifically, represents a rational and novel drug target against amebiasis.

2. Materials and methods

2.1. Organisms, cultivation, and chemicals

Trophozoites of *E. histolytica* clonal strains HM-1: IMSS cl 6 and G3 (Bracha et al., 2006) were maintained axenically in Diamond's BI-S-33 medium at 35.5 °C as described previously (Diamond et al., 1978). Trophozoites were continuously maintained in mid-log phase after inoculation of one-thirtieth to one-twelfth of the total culture volume. *Escherichia coli* BL21 (DE3) strain was obtained from Invitrogen (Carlsbad, CA, USA). Magnesium-free ATP was procured from DiscoverX (Fremont, CA, USA). Ni^{2+}-NTA agarose was procured from Novagen (Darmstadt, Germany). Lipofectamine and geneticin (G418) were procured from Invitrogen. Chemicals to evaluate metals in PanK activity assay were procured from Wako (Tokyo, Japan). All other chemicals of analytical grade were procured from Sigma-Aldrich (Tokyo, Japan) unless otherwise stated.

2.2. Production of PanK gene-silenced strain

In order to construct a plasmid for epigenetic gene silencing of *E. histolytica* (Bracha et al., 2006; Zhang et al., 2011) *PanK* (*EhPanK*), a fragment corresponding to a 430 bp 5′ open reading frame of *EhPanK* gene was amplified by PCR from cDNA using the following primer set (sense primer, 5′-CAG**AGGCCT**ATGTCTCAACCATCCCATTCT-3′ and antisense primer, 5′-AAT**GAGCTC**TCTGAAGATTACCAATCCCAT AAA-3′). These oligonucleotides contained StuI and SacI restriction sites (shown in bold). The amplified product was digested with StuI and SacI, and ligated into the StuI and SacI double digested psAP2-Gunma construct (Husain et al., 2011) to synthesize a *EhPanK* gene silencing plasmid. The G3 strain trophozoites were transformed with an empty vector as a control and the silencing plasmid was transfected by liposome-mediated transfection as previously described (Nozaki et al., 1999). Transformants were initially selected in the presence of 1 μg/mL geneticin gradually increased to 10 μg/mL.

2.3. Reverse transcriptase PCR

RNA was extracted from approximately 1×10^6 trophozoites of *EhPanK* gene silenced (PanK gs) and control transformant strains using TRIzol reagent (Ambion, Life Technologies) as previously described (Chomczynski and Mackey, 1995). DNase treatment was performed using DNase I (Invitrogen) to exclude genomic DNA. RNA quantity was determined by measuring the absorbance at 260 nm with a NanoDrop ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Approximately one μg total RNA was used for cDNA synthesis using First-Stand cDNA Synthesis (Superscript® III, Invitrogen) with reverse transcriptase and oligo (dT) primers according to manufacturer's instructions. The cDNA product was diluted 10-fold and PCR reactions were carried out in 50 μl, using the primer pair (Sense primer, 5′-ATGTCTCAACCATCCCATTC-3′ and antisense primer, 5′-TTACATTA GTTCTTCTTCATACTC-3′). The PCR conditions were: 98 °C, 10 s; 55 °C, 1 min; and 72 °C, 1 min; 20–25 cycles. The PCR products obtained were resolved by agarose gel electrophoresis.

2.4. Quantitative real-time (qRT) PCR

Relative levels of steady-state mRNA of the following genes were measured using qRT-PCR: *PanK* (EHI_183060), bifunctional phosphopantothenate-cysteine ligase/decarboxylase (*PPCS-PPCDC*, EHI_164490), phosphopantetheine adenylyltransferase (*PPAT*, EHI_006680), dephospho CoA kinase 1 and 2 (*DPCK1,* EHI_040840; and *DPCK2*, EHI_155780), and RNA polymerase II gene (EHI_056690) as a control. Each 20 μL reaction contained 10 μL 2X Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.6 μL each of 10 μM sense and antisense primers, 5 μL 10x diluted cDNA, and nuclease-free water. PCR was performed using the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: enzyme activation at 95 °C for 20 s, followed by 40 cycles of denaturation at 95 °C for 3 s and annealing-extension at 60 °C for 30 s. All reactions were carried out in triplicate, including cDNA-minus controls. The amount of the steady-state mRNA of each target gene was determined by the ΔCt method with RNA polymerase II as a reference gene (Livak and Schmittgen, 2001). The mRNA expression level of each gene in the transformant was expressed relative to that in the control transfected with psAP2.

2.5. Production of whole lysates from E. histolytica trophozoites

Approximately 1×10^6 trophozoites were harvested 48 h after initiation of culture and washed with 2% glucose in 1X phosphate buffer saline (PBS) three times. Cells were counted and resuspended in 500 μL homogenization buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 50 mM NaCl) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mg/mL E-64 (Peptide Institute, Osaka, Japan). Cells were disrupted mechanically by a Dounce homogenizer and kept on ice for 30 min with intermittent vortexing followed by centrifugation at 500 × g for 30 min at 4 °C for removing the insoluble cellular debris. The supernatant, representing total cell lysate, was carefully collected. Protein concentrations were spectrophotometrically determined by the Bradford method using bovine serum albumin as a standard as previously described (Bradford, 1976).

2.6. Enzyme assays and quantitation of CoA in cell lysates

PanK and DPCK activities in the cell lysate were measured with coupled assays using ADP Hunter™ Plus Assay kit (DiscoverX, US) according to the manufacturer's instructions. Briefly, fluorescence intensities were continuously measured to estimate the formation of resorufin at 37 °C by excitation at 530 nm and emission at 590 nm in a 25 μL reaction mixture [10 mM $MgCl_2$, 15 mM HEPES, 20 mM NaCl, 1 mM EGTA, 0.02% tween 20, 0.1 mg/mL β-globulin, 2 mM pantothenate or 2 mM dephospho CoA for PanK or DPCK, respectively, 0.1 mM ATP, 2 μL of cell lysate (~5 μg protein)]. Kinetic data were estimated by curve fitting with the Michaelis–Menten equation using GraphPad Prism (GraphPad Software Inc., San Diego, USA). This experiment was repeated three times in triplicate with proteins isolated from two independent extractions, and kinetic values are presented as the means ± S.E. for three independent kinetic assays.

Concentrations of CoA in the cell lysate were measured using the CoA assay kit (BioVision, CA, USA) according to manufacturer's instructions. CoA at 0.05–1 nmole was used to produce a standard curve to determine the amounts of CoA in lysates. Experiment was conducted in triplicate, and repeated three times on three different days.

2.7. Monitoring of growth kinetics

Trophozoite cultures were continuously maintained in mid-log phase as described previously, and placed on ice for 5 min to detach

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