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RESEARCH ARTICLE

Inhibition of choline kinase as an antiamoebic approach in *Entamoeba histolytica* infection

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ABSTRACT

Entamoeba histolytica is the parasite responsible for amoebiasis, which can result in amoebic colitis or amoebic liver abscess. Metronidazole has been the conventional treatment for intestinal amoebiasis, but concerns regarding resistance have emerged due to the identification of resistance pathways in *E. histolytica*. This study investigates a novel anti-amoebic approach targeting the CDP-choline pathway. Inhibition studies were conducted using potential choline kinase (CK) inhibitors to inhibit the EhCK enzyme, and RNA interference was employed to knock down the EhCK gene. K_m and V_{max} of purified EhCK and hCKα2 proteins were determined by pyruvate kinase-lactate dehydrogenase (PK-LDH) coupled assay. The IC_{50} values for EhCK and hCKα2 were determined with several commercial CK inhibitors. Selected inhibitors were incubated with *E. histolytica* trophozoites for 48 hours to determine the EC_{50} for each inhibitor. Silencing of gene encoding EhCK was carried out using duplex siRNA and the gene expression level was measured by real-time qPCR. Based on the IC_{50} values, three of the inhibitors, namely CK37, flavopiridol and H-89 were more potent against EhCK than hCKα2. Trophozoites growth inhibition showed that only HDTAB, H-89 and control drug metronidazole could penetrate and induce cell death after 48-hour incubation. siRNA concentration of 10 µg/mL was used for the transfection of positive control GAPDH, EhCK, and non-targeting GFP siRNAs. RNAi experiment concluded with positive control GAPDH downregulated by 99% while the level of EhCK mRNA was downregulated by 47%. In this study, potential inhibitors of EhCK and siRNA have been identified, paving the way for further refinement and testing to enhance their potency against EhCK while sparing hCK. The utilization of these specific inhibitors and siRNA targeting EhCK represents a novel approach to impede the growth of *E. histolytica* by disrupting its phospholipid synthesis pathway.

Keywords: *Entamoeba histolytica*; choline kinase inhibitors; anti-amoebic approach; siRNA.

INTRODUCTION

Amoebiasis is a tropical disease caused by the protozoan parasite *Entamoeba histolytica*, often overlooked despite its significant consequences. This microorganism is the second most virulent protozoa, next only to *Plasmodium falciparum*, and it is a principal contributor to diarrheal diseases worldwide, particularly affecting people residing in impoverished regions of developing nations (Shrivastav *et al*., 2020). According to the World Health Organization (WHO), approximately 500 million people worldwide are infected with *Entamoeba* spp., with *E. histolytica* responsible for around 50 million cases (Martínez-Castillo *et al*., 2021).

In developing countries, amoebiasis poses a significant public health challenge due to inadequate sanitation practices that facilitate the faecal-oral transmission of the *E. histolytica* parasite through contaminated food and drinking water. This can lead to the onset of severe symptoms including fever, chills, abdominal discomfort, and the presence of bloody or mucous diarrhea. In some regions of Central and South America, Africa, and Asia, up to 40% of the population is infected with amoebic dysentery due to poor hygiene and sanitation practices (Negrut *et al*., 2020).

For the past 50 years, metronidazole has been the recommended treatment for amoebiasis. While there is currently no clinical evidence of metronidazole resistance in *E. histolytica*, *in vitro* experiments have indicated the potential existence of metronidazole-resistant trophozoites, highlighting the need for further investigation (Samarawickrema *et al*., 1997; Wassmann *et al*., 1999). This study investigates the inhibition of the CDP-choline pathway in *E. histolytica* as an alternative approach to combat amoebiasis. Inhibition of choline kinase, the initial enzyme in the CDP-choline pathway, has previously been identified as an anti-malarial strategy in the parasite *P. falciparum* (Ancelin & Vial, 1986; Choubey *et al*., 2007; Zimmerman *et al*., 2013).

There has been a recent surge in interest regarding the use of eukaryotic choline kinase inhibitors (CKIs) for the treatment of parasites. CK is the first enzyme in the CDP-choline pathway, responsible for synthesizing PC, the major (40 to 50% of total phospholipids) and essential phospholipid in *P. falciparum* membrane

(Choubey *et al*., 2007; Serrán-Aguilera *et al*., 2016). The potential use of choline analogs to inhibit *de novo* phosphatidylcholine synthesis has been suggested as a prospective treatment for malaria, including drug-resistant strains (Ancelin *et al*., 2003). Therefore, inhibition of *P. falciparum* CK (PfCK) has been considered a promising antimalarial strategy. PfCK is expressed at higher levels during the parasite's growth phases (Choubey *et al*., 2006) and becomes less viable after CK inhibition (Choubey *et al*., 2007). MN58b, a second-generation choline kinase inhibitor (CKI), and RSM-932A, a third-generation CKI, have demonstrated the ability to inhibit PfCK with minimal toxicity to humans. These inhibitors have been observed to reduce parasitemia by interfering with the maturation and invasion processes of *P. falciparum* (Zimmerman *et al*., 2013). These findings strongly suggest that a similar approach can be used to develop new anti-amoebiasis strategies by inhibiting choline/ethanolamine kinases.

In this study, gene regulation was achieved through the utilization of small interfering RNA (siRNA) to induce mRNA degradation, with the objective of investigating the feasibility of silencing the EhCK gene. To achieve this, the siRNA soaking method was used because of its short experimental period, short-term, and immediate suppression effect. Due to the ease of design and direct incorporation into *E. histolytica*, siRNAs were the preferred dsRNA to be used in this study.

The advent of RNAi technology has revolutionized our understanding of gene regulation and metabolic pathways in organisms. By targeting gene expression at the mRNA level, it is possible to reduce protein levels in the cell, causing a disruption of protein complexes. Silencing EhCK will enable us to determine if inhibition is achievable at both the mRNA and protein levels. Although RNAi-based therapy offers advantages in terms of specificity and high potency, delivering the RNAi molecules into diseased tissues and cells and ensuring their stability remains challenging (Lu *et al*., 2005). Currently, RNAi therapeutics are available for targeting organs, tumors, and infectious diseases, with some undergoing Phase III development (Bobbin & Rossi, 2016). With improvements in the delivery method and reduction in off-target effects, RNAi therapeutics have the potential to be applied to a wide range of diseases, including parasitic diseases like amoebiasis (Kolev *et al*., 2011; Mueller *et al*., 2014).

MATERIALS AND METHODS

Organisms

The *E. histolytica* strain HM-1:IMSS trophozoites (Courtesy of Dr. Lim Boon Huat, Universiti Sains Malaysia and Dr. Alfonso Olivos-García of Universidad Nacional Autónoma de México) were cultured axenically at 36°C in TYI-S-33 medium (Diamond *et al*., 1978), which was supplemented with 10% heat-inactivated bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 3% 1x Diamond vitamin-Tween 80 (Sigma, St. Louis, MO, USA). The medium was refreshed every 48 hours to maintain optimal cell growth conditions.

For plasmid propagation and protein expression, *Escherichia coli* XL1-Blue and BL21 (DE3) strains were used. These bacterial cells were grown in Luria Bertani (LB) (Merck, Darmstadt, Germany) broth and were supplemented with 100 µg/mL ampicillin (Merck, Darmstadt, Germany) as and when required.

Inhibition of *E. histolytica* trophozoites with inhibitors

The experiment involved treating *E. histolytica* cells in a 96-well plate for 48 hours. Each well was seeded with roughly 5×10^4 trophozoites, which were then incubated in a zipper bag containing an anaerobic pack (AnaerogenTM 2.5 L, UK) for 1-2 hours at 37°C. Following this incubation period, different serial dilutions of drug concentrations (from 3.05 µM to 195.31 µM), including 2-amino-1-butanol, CK37, HDTAB, flavopiridol, H-89, thiamine HCl, and hemicholinium-3 (HC-3), were added to their respective wells (Razak *et al*., 2014). A positive and negative controls were included using metronidazole and DMSO, respectively. The plate was then left to incubate for an additional 48 hours at 37°C with the anaerobic pack before measuring cell viability via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay (Mosmann, 1983). For this assay, viable trophozoites after 48 hours, were settled by centrifuging the plate at 50×*g* for 2 minutes. The TYI-S-33 medium was replaced with 100 µL of DMEM, and 10 µL of MTT solution was added to each well. The plate was incubated at 37°C in the dark with anaerobic pack for 2 hours. Afterward, the purple formazan was solubilized by adding 100 µL of DMSO to each well and pipetting thoroughly. Absorbance of the solubilized formazan was measured at 570 nm.

EhCK Genes Cloning and Expression Vectors Construction

The genomic DNA of *E. histolytica* HM-1:IMSS was isolated using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The open reading frame (ORF) of the EhCK gene was then amplified from the genomic DNA using the Platinum® Pfx DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with forward primers 5'- GGGAATTCCATATGACAGTGGTACAAAG-3' and reverse primers 5'-CGCGGATCCTTAAAGAATCTTTTTTAATTCG-3'. Restriction enzyme sites (indicated by underlining) were introduced to facilitate cloning. PCR was carried out using a three-step cycling protocol with an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, primer annealing at 40°C for 30 s, and extension at 68°C for 1.5 min. The final extension was performed at 68°C for 7 min. All amplicons were analyzed in a 1% agarose gel and purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. To express EhCK as glutathione-S-transferase (GST) fusion proteins, EhCK was cloned into pGEX-RB (Brundiers *et al*., 1999) at the restriction enzyme sites. The recombinant plasmids were screened by restriction enzyme digestion and confirmed by DNA sequencing. The recombinant plasmids were designated as pGEX RB-EhCK.

EhCK and hCKα2 Expression and Purification in *E. coli* Expression System

The recombinant plasmids pGEX RB-EhCK and pGEX RB-hCKα2 were introduced into *E. coli* BL21 (DE3) strain via heat shock method. For expression of GST-EhCK, previously optimized parameters by Chang (2012) were followed. The induction was carried out at 27°C for 16 hours using 1 mM IPTG (Sigma, St. Louis, MO, USA) when the OD600nm of the culture was between 0.7-0.8. The expression of GST-hCKα2 followed the optimized parameters established by Wong (2012) and was induced with 0.5 mM isopropyl β- d-1-thiogalactopyranoside (IPTG) for 16 hours at 25°C.

The bacterial culture was harvested by centrifugation at 10,000 × *g* for 15 minutes at 4°C. Bacterial cells from a 50 mL culture were resuspended in lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10% glycerol, and 5 mM β-mercaptoethanol, supplemented with 1× protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The suspension was sonicated for 4 minutes at 0.5 sec on/off pulse and then centrifuged at 15,000 × *g* for 20 minutes. The supernatant containing the GST-tagged proteins was affinity purified using GST•Bind™ Resin (Thermo Fisher Scientific, Waltham, MA, USA). The resin was washed six times with washing buffer (lysis buffer without EDTA and β-mercaptoethanol) to remove non-specific binding. The GST tag was removed via thrombin cleavage with 1 NIH unit/mL thrombin for 16 hours prior to elution. The homogeneity of purified protein was analyzed by SDS-PAGE, and the protein concentration was determined by the Bradford method (Bradford, 1976). All steps were performed in a cold room or on ice, and the purified proteins were stored at -80°C until use.

Enzymatic Assay

CK activities of EhCK and hCKα2 were spectrophotometrically measured using a pyruvate kinase-lactate dehydrogenase coupled assay (Malito *et al*., 2006). The kinetic reaction was monitored by measuring the rate of absorbance decrease at 340 nm due to NADH oxidation. The standard assay was performed in a 1 mL reaction mixture containing 100 mM Tris-HCl (pH 8.0 for EhCK and pH 7.5 for hCKα2), 150 mM KCl, and either 12 mM MgCl2 or MnCl2, depending on the experiment. Additionally, 10 mM ATP, 0.5 mg bovine serum albumin, 0.5 mM phosphoenolpyruvate, 0.25 mM NADH, 4 units lactate dehydrogenase, and 5 units pyruvate kinase were added to the reaction mixture. For EhCK, 3 µg of protein and 5 mM of substrate choline were used in each measurement, while for hCKα2, 2 µg of protein and 5 mM of choline were used. The reaction mix was supplemented with an inhibitor, warmed to 37°C, and the substrate was added once the absorbance reading reached a stable level. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol ADP per minute. The kinetic data were analyzed using GraphPad Prism software (GraphPad Software) and fitted with non-linear regression using the Michaelis-Menten equation.

siRNA silencing experiment

The siRNA silencing experiment was conducted in 24-well plates, each containing a working volume of 1 mL/well. *E. histolytica* trophozoites (5×10^4) were seeded in each well and allowed to incubate for 24 hours at 37°C until reaching 50% confluency. Then, siRNA at a concentration of 10 µg/mL was added to the wells, while a separate well containing GFP siRNA at the same concentration was used as a negative control. The plate was incubated in an anaerobic environment using an airtight zipper bag containing an anaerobic pack (Anaerogen™ 2.5 L, UK) for 16 hours at 37°C. Following incubation, total RNA was extracted from the trophozoites to quantify the gene expression levels using real-time PCR.

Gene expression analysis using ΔΔCT method

The method for calculating gene expression in this study was based on a technique recommended by Dharmacon (Haimes & Kelley, 2014). First, the expression of the siRNA-treated gene was normalized to that of the reference gene, Actin, within the same sample to yield ΔC_T. ΔC_T for each replicate was transformed exponentially to ΔC_T Expression using the formula $2^{-\Delta\Delta CT}$. The average ΔC_T Expression was calculated, and the standard deviation was determined. The mean expression of the target gene was normalized to the expression of the same gene in the sample treated with non-targeting GFP siRNA to obtain ΔC_T Expression. Finally, percent knockdown (%KD) was calculated using the formula $(1-\Delta\Delta C_T)\times100$.

Data analyses

The data were analysed and presented in line and bar graphs. Statistical significance was determined using a Student's t-test in IBM® SPSS® Statistics Version 22.

RESULTS

Enzyme kinetics of *E. histolytica* choline kinase and human choline kinase α2 with magnesium and manganese cofactors

In this study, the Michaelis-Menten constant of EhCK was determined using a PK-LDH coupled assay with choline and ATP as substrates. EhCK showed a hyperbolic pattern of Michaelis-Menten equation in both magnesium and manganese cofactor conditions. With magnesium as the cofactor, the K_m and V_{max} values for choline were 0.20 ± 0.044 mM and 2.13 ± 0.14 U/mg, respectively (Figure 1A), while the V_{max} significantly increased to 34.12 ± 1.87 U/mg with manganese as the cofactor, with a corresponding K_m value of 0.12 ± 0.030 mM (Figure 1B). Furthermore, the enzyme activity of EhCK was measured with magnesium and manganese as cofactors for ATP substrate, resulting in V_{max} and K_m values of 3.02 ± 0.37 U/mg and 10.85 ± 2.33 mM with magnesium (Figure 2A), and 48.98 ± 2.14 U/mg and 1.61 ± 0.26 mM with manganese, respectively (Figure 2B). These results suggest that the use of manganese as a cofactor could significantly increase the V_{max} of EhCK for both choline and ATP substrates.

Based on the previous study by Chang (2012), magnesium was selected as the cofactor for hCKα2 in this study. The enzyme activity of hCKα2 was assayed, and a V_{max} value of 71.4 ± 1.54 U/mg and a K_m of 0.12 ± 0.012 mM were determined for choline (Figure 3A). Meanwhile, for ATP substrate, the V_{max} and K_m values were found to be 113.6 ± 5.13 U/mg and 0.67 ± 0.085 mM, respectively (Figure 3B).

Inhibition study of various compounds on EhCK and hCKα2

In this study, seven potential inhibitors were evaluated for their ability to inhibit EhCK activity. Based on the results, two of them, 2-amino-1-butanol and thiamine HCl, were shown to be ineffective as inhibitors, as demonstrated by their non-dose-dependent curve equation and an IC_{50} value that was beyond the tested concentration range. When tested at the highest concentration tested (900 µM), 2-amino-1-butanol only caused a marginal 9.1% decrease in EhCK activity. Furthermore, thiamine HCl at 125.44 µM concentration unexpectedly resulted in a 10.85% increase in EhCK activity

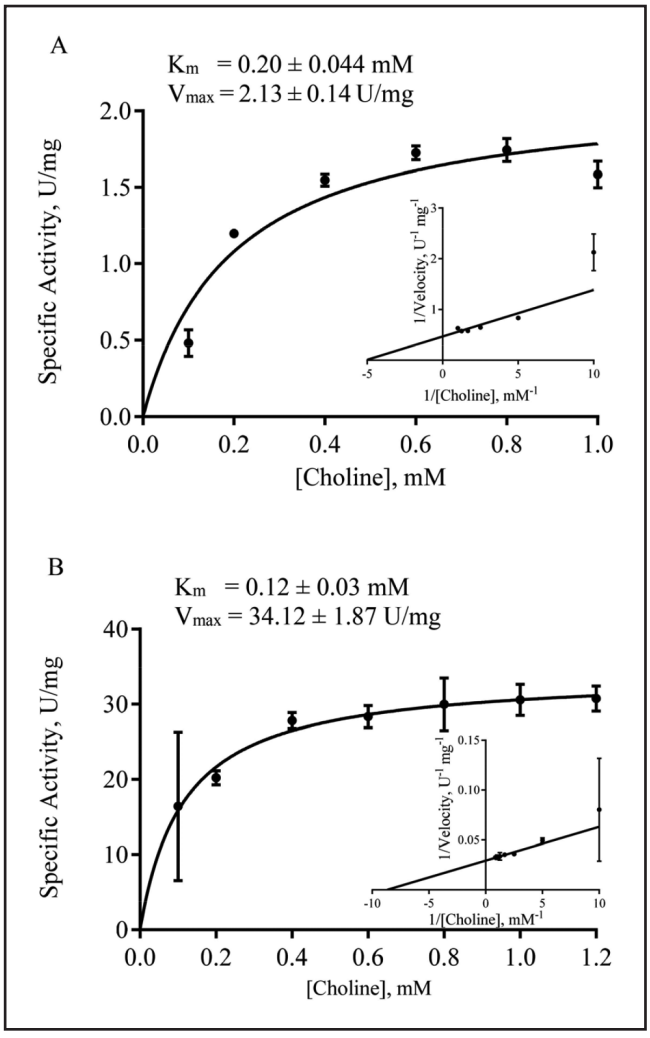

Figure 1. Determination of EhCK Michaelis-Menten constants for choline with (A) magnesium and (B) manganese as the cofactor. Inner graph: Lineweaver-Burk double reciprocal plot. Results plotted are mean and S.D. of triplicate experiments.

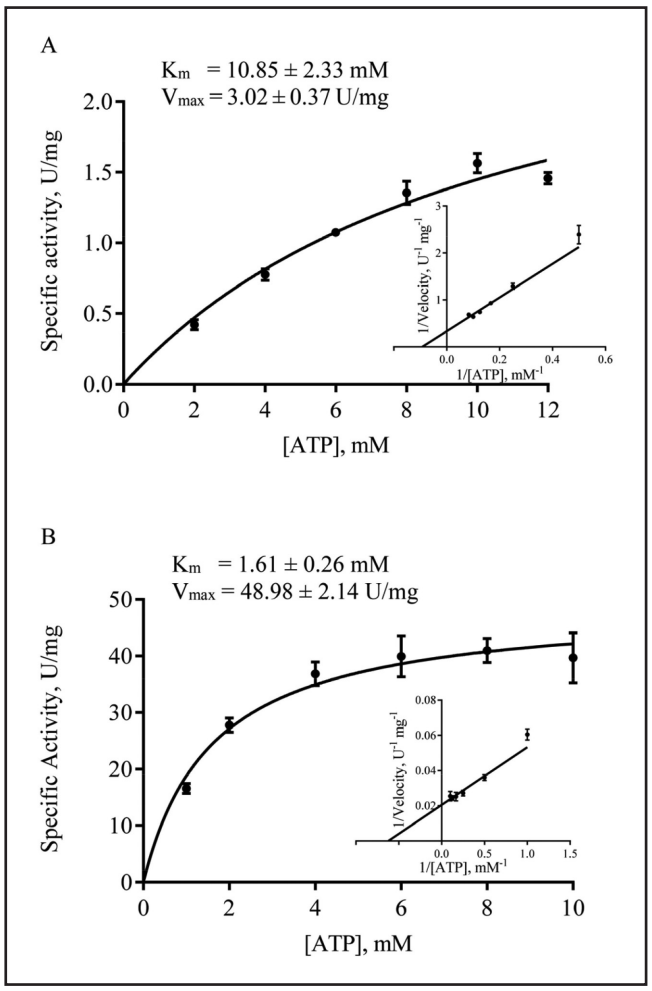

Figure 2. Determination of EhCK Michaelis-Menten constants for ATP with (A) magnesium and (B) manganese as the cofactor. Inner graph: Lineweaver-Burk double reciprocal plot. Results plotted are mean and S.D. of triplicate experiments.

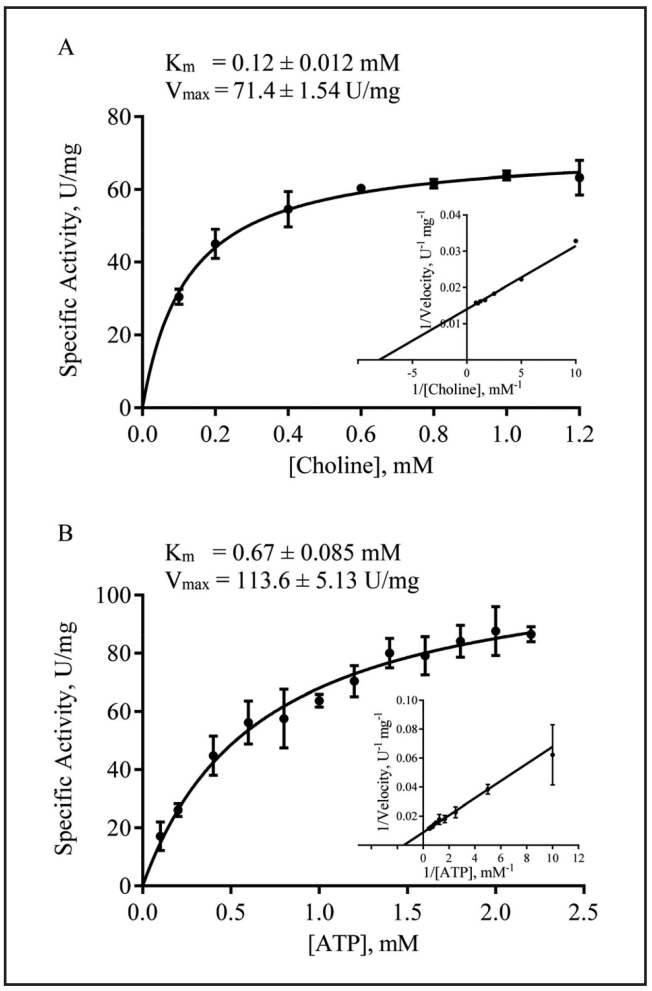

Figure 3. Determination of hCKα2 Michaelis-Menten constants for (A) choline and (B) ATP with magnesium as a cofactor. Inner graph: Lineweaver-Burk double reciprocal plot. Results plotted are mean and S.D. of triplicate experiments.

compared to the control. Hence, these inhibitors were deemed unsuitable for further testing.

Inhibition of EhCK and hCKα2 by flavopiridol

Flavopiridol is a CDK-specific ATP analogue inhibitor that targets *P. falciparum* protein kinase 5 (Graeser *et al*., 1996). It was found to effectively inhibit EhCK with magnesium as cofactor (IC_{50} = 45.51 µM ± 15.50 µM), while the IC_{50} was higher when manganese was used (184.60 µM ± 31.40 µM). The inhibitor also showed lower potency on hCKα2 (IC_{50} = 297.0 µM ± 80.50 µM) (Figure 4). Further testing of flavopiridol on *E. histolytica* trophozoites was conducted based on these findings.

Inhibition of EhCK and hCKα2 by choline kinase α inhibitor (CK37)

CK37 was found to be a potent inhibitor for both hCKα2 and EhCK, with the most effective inhibition observed for EhCK using magnesium as cofactor. The IC_{50} values for EhCK were 60.64 µM ± 14.67 µM and 111.80 µM ± 31.25 µM for magnesium and manganese cofactors, respectively. The IC_{50} for hCKα2 was 68.41 µM ± 12.08 µM.

Inhibition of EhCK and hCKα2 by H-89 dihydrochloride hydrate

H-89 is a well-known protein kinase A inhibitor that competes with substrate ATP for enzyme ATP binding site. In this study, H-89 was found to be an effective inhibitor of EhCK with IC_{50} values of 100.60 µM ± 17.41 µM and 129.10 µM ± 13.56 µM in the presence of magnesium and manganese, respectively. Unlike PfCK, H-89 inhibited EhCK despite not affecting hCKα2 to a large extent. The compound exhibited a lower potency in inhibiting hCKα2, with an IC_{50} of 392.10 µM ± 36.62 µM. H-89 was also selected for further testing on *E. histolytica* trophozoites.

Inhibition of EhCK and hCKα2 by hemicholinium-3 (HC-3)

In this study, HC-3 inhibited EhCK with an IC_{50} of 256.70 µM ± 21.74 µM using magnesium as a cofactor, and less effectively with manganese as cofactor with an IC_{50} of 654.90 µM ± 42.06 µM. On the other hand, HC-3 exhibited the highest potency towards hCKα2 with an IC_{50} of 2.07 µM ± 0.22 µM. The potency of HC-3 followed the order of hCKα2 > EhCK with magnesium > EhCK with manganese.

Inhibition of EhCK and hCKα2 by hexadecyltrimethylammonium bromide (HDTAB)

HDTAB dose-dependent inhibition of EhCK produced IC_{50} of 69.22 µM ± 9.21 µM (magnesium cofactor) and 156.20 µM ± 18.19 µM (manganese cofactor). HDTAB was more potent in inhibiting hCKα2 (IC_{50} of 29.38 µM ± 2.65 µM). The highest potency is in hCKα2, followed by EhCK in magnesium and EhCK in manganese. HDTAB was selected for further investigation on *E. histolytica* trophozoites based on its previous inhibition of PfCK (Choubey *et al*., 2007; Razak *et al*., 2014).

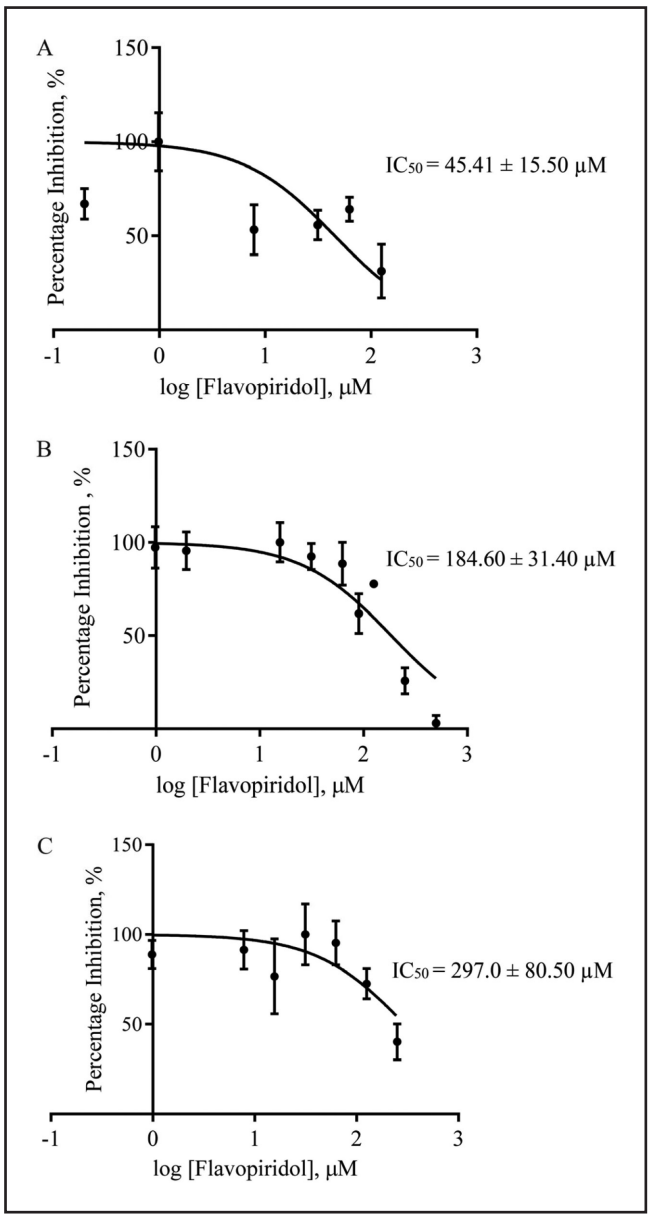

Figure 4. Inhibition of EhCK and hCKα2 by flavopiridol. IC_{50} determination of flavopiridol on EhCK with (A) magnesium and (B) manganese cofactor. IC_{50} determination of flavopiridol on hCKα2 (C). Results plotted are mean and S.D. of triplicate experiments.

Table 1 shows the summary of IC_{50} values of inhibitors for EhCK and hCKα2 with Mg^{2+} or Mn^{2+} cofactors.

Determination of *E. histolytica* trophozoites cell viability after inhibitor treatment

In this study, the growth inhibition of *E. histolytica* trophozoites by four different inhibitors was investigated. Trophozoites were seeded at 5×10^4 per well, with the initial seeding density determined by microscopic observation. Trophozoites were active and viable before treatment, and inhibitors were incubated with the trophozoites for 48 hours, with metronidazole used as the positive control. The effective concentration (EC_{50}) was determined using GraphPad Prism 7, with the highest percentage viability set to 100 and the lowest to 0.

Metronidazole induced trophozoite death after 48 hours of incubation. Viability was only affected at concentrations of 3.05 µM to 195.32 µM, while lower dosages of 0.38 µM and 0.75 µM had no impact on viability. The EC_{50} value for metronidazole was 1.73 µM ± 0.33 µM (Figure 5A).

Flavopiridol was found to cause cell lysis at higher concentrations (>3.05 µM) of flavopiridol, while trophozoites remained viable at low concentrations (0.8 µM and 1.5 µM). Viability increased at concentrations between 48.83 µM to 195.32 µM. The EC_{50} of flavopiridol was 3.91 µM ± 1.76 µM (Figure 5B).

H-89, a protein kinase A inhibitor, caused cell lysis in high dosages (97 µM – 195 µM) after prolonged incubation for 48 hours. The EC_{50} of H-89 was 32.44 µM ± 5.05 µM (Figure 5C).

HDTAB, an inhibitor of choline kinase, showed greater potency in inhibiting hCKα2, but its effect on trophozoite viability was also investigated, given its previously reported success in inhibiting choline kinase of *P. falciparum* (Choubey *et al*., 2007; Razak *et al*., 2014). At lower dosages (6.1 µM – 12 µM), trophozoites did not undergo lysis but showed signs of stress, such as rounding up. The resulting EC_{50} for HDTAB was 47.40 µM ± 7.22 µM (Figure 5D).

Table 2 provides a summary of the EC_{50} values of metronidazole, HDTAB, and H-89 on *E. histolytica* trophozoites. Metronidazole exhibited the highest potency among the compounds tested, followed by flavopiridol. H-89 and HDTAB exhibited lower potency.

Determination of gene expression after siRNA treatment

Transfection with both the positive control GAPDH siRNA and the EhCK siRNA led to downregulation of gene expression. Microscopic examination revealed that the morphology of the trophozoites remained unchanged following transfection with siRNAs targeting EhCK and GAPDH, as well as the non-targeting GFP siRNA. Prior to RNA extraction, trophozoites were approximately 70% confluent

Table 1. IC_{50} values of selected inhibitors for EhCK and hCKα2 with Mg^{2+} or Mn^{2+} as cofactor. [*] and bold indicate inhibitors that were selected for treatment of *E. histolytica* trophozoites

Inhibitors	Type of inhibitors	IC_{50} (µM)		
		EhCK (Mg^{2+})	EhCK (Mn^{2+})	hCKα2 (Mg^{2+})
CK37	Choline competitive inhibitor (Clem *et al.*, 2011).	60.64 ± 14.67	111.80 ± 31.25	68.41 ± 12.08
***Flavopiridol**	ATP competitive inhibitor (Sedlacek, 2001). – chosen for further study because it showed effective in inhibiting EhCk, but lower potency on hCKα2	45.51 ± 15.50	184.60 ± 31.40	297.0 ± 80.50
***H-89**	ATP competitive inhibitor (Murray, 2008). – chosen for further study because it showed effective in inhibiting EhCk, but lower potency on hCKα2	100.60 ± 17.41	129.10 ± 13.56	392.1 ± 36.62
HC-3	Choline competitive inhibitor (Hamza *et al.*, 1983).	256.70 ± 21.74	654.90 ± 42.06	2.07 ± 0.22
***HDTAB**	Choline competitive inhibitor (Choubey *et al.*, 2007; Razak *et al.*, 2014). – chosen for further study based on its previous inhibition of PfCK.	69.22 ± 9.21	156.20 ± 18.19	29.38 ± 2.65

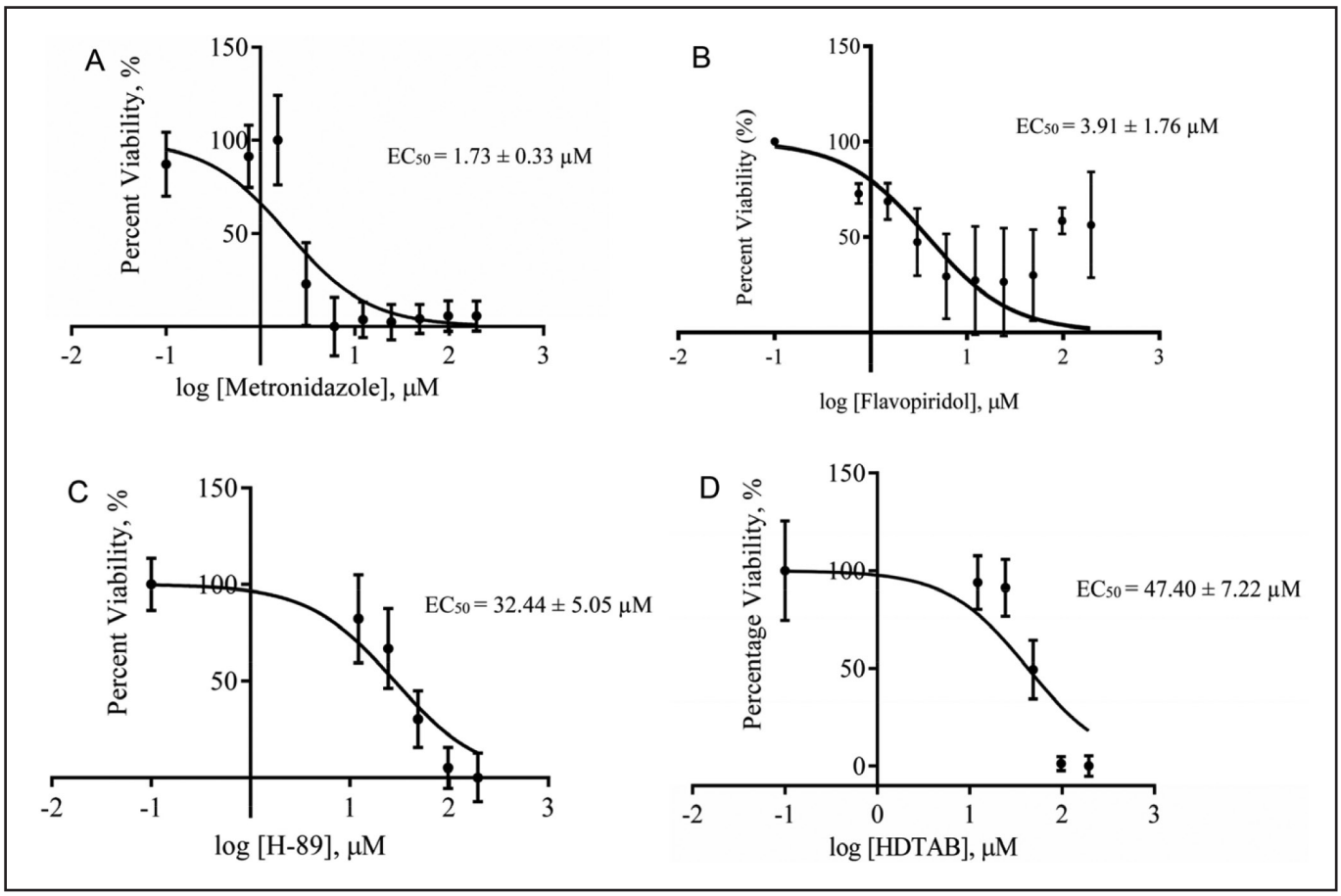

Figure 5. (A) Determination of EC_{50} of 48-hour metronidazole treatment on *E. histolytica*. (B) Determination of EC_{50} of 48-hour flavopiridol treatment on *E. histolytica*. (C) Determination of EC_{50} of 48-hour H-89 treatment on *E. histolytica*. (D) Determination of EC_{50} of 48-hour HDTAB treatment on *E. histolytica*.

Table 2. The EC_{50} of inhibitors on *E. histolytica* trophozoites after 48-hour incubation

Inhibitors	EC_{50} (µM)
Metronidazole	1.73 ± 0.33
Flavopiridol	3.91 ± 1.76
H-89	32.44 ± 5.05
HDTAB	47.40 ± 7.22

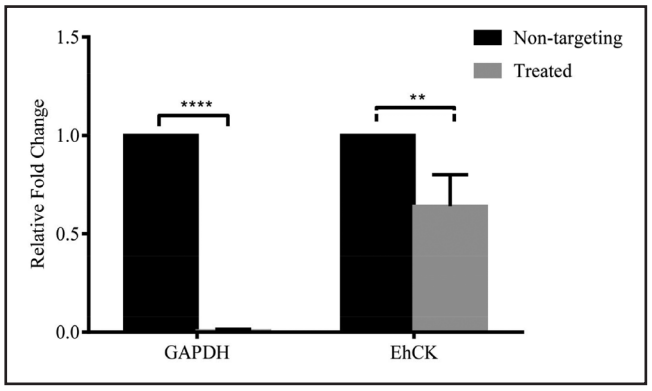

Figure 6. Relative gene expression after silencing experiment. Data and error bars for S.D were plotted based on results from 3 independent experiments. Asterisks (*) show significant difference based on Student's t-test ($p < 0.05$).

and motile. As shown in Figure 6, the positive control GAPDH siRNA resulted in a significant downregulation of the GAPDH gene expression by approximately 99%. The EhCK siRNA resulted in a 47% knockdown of gene expression. Statistical analysis using a student's t-test showed that the difference in gene expression was statistically significant ($p < 0.05$) for both GAPDH ($p < 0.0001$) and EhCK ($p = 0.0006$) when compared to the negative control.

DISCUSSION

For more than 50 years, metronidazole has been the preferred treatment for amoebiasis. Nevertheless, there are apprehensions regarding the potential emergence of resistance in clinical strains of *E. histolytica* due to prolonged use of metronidazole (Gonzales *et al*., 2019). Research findings have indicated that laboratory-induced metronidazole-resistant strains of *E. histolytica* exhibit a resistance-related pathway (Samarawickrema *et al*., 1997). Due to this, researchers are currently exploring alternative pathway targets for treating amoebiasis. This study investigated the CDP-choline pathway as a potential antiamoebic target.

In this study, competitive inhibitors of ATP and choline substrate were chosen. Many small molecule CKα inhibitors designed target the choline binding site. The choline binding site consists of a deep hydrophobic groove with conserved aromatic residues, surrounded by negatively charged conserved residues. This structure guides the choline substrate into the active site, where the positively charged quaternary amine is stabilized by the hydrophobic aromatic residues (Malito *et al*., 2006).

Ancelin and Vial (1986) used 2-amino-1-butanol to inhibit PfEK, confirming its specificity for ethanolamine substrate by inhibiting

phosphatidylethanolamine (PtdEtn) biosynthesis in *Plasmodium*. Similar results were observed with EhCK, as 2-amino-1-butanol did not inhibit its activity. Thiamine HCl and choline are methyl-rich substrates but showed no direct relationship with either EhCK or hCKα2, as they did not exhibit antagonist or synergistic effects on enzyme activity. Miltefosine, an anti-leishmaniasis drug, has anti-protozoal properties against *E. histolytica* by inhibiting choline phosphate cytidyltransferase (CCT) in the CDP-choline pathway (Sundar & Olliaro, 2007). However, this study found that miltefosine does not inhibit EhCK and is not competitive with choline substrate, possibly because its structure is more similar to the substrate of CCT- phosphocholine (Choubey *et al*., 2007).

Other compounds tested (CK37, flavopiridol, H-89, HDTAB, and HC-3) could inhibit EhCK and hCKα2 *in vitro*. CK37, flavopiridol, and H-89 demonstrated higher potency as inhibitors of EhCK when Mg^{2+} was present, while HC-3 and HDTAB showed greater potency in inhibiting hCKα2. However, the potency of these inhibitors decreased in EhCK when Mn^{2+} was used as a cofactor, possibly due to higher EhCK activity with Mn^{2+}. It's important to note that measuring IC_{50} using non-physiological cofactors may distort true inhibitor potency (Knight & Shokat, 2005).

HC-3 exhibited the highest potency among the tested compounds with an IC_{50} of 2.07 µM ± 0.22 µM. HDTAB, which shares structural similarities with both choline and miltefosine, was suggested to inhibit PfCK through competitive inhibition with choline (Choubey *et al*., 2007). While HDTAB was found to inhibit EhCK in vitro and induce cell lysis in culture, it remains unclear whether the cell lysis was induced via inhibition of EhCK or ATP synthase.

Among all the inhibitors, only five (CK37, flavopiridol, H-89, HC-3, and HDTAB) were tested in trophozoite cultures. CK37 and HC-3 had no effect on *E. histolytica* viability or trophozoite morphology after 48 hours of incubation. The lack of inhibition might be attributed to the cell permeability of the inhibitors, which depends on their hydrophobic and lipophilic nature, charge, and phosphorylation (Mohan *et al*., 2013). Charged and hydrophobic compounds are generally not cell-permeable due to hydrogen bonds or lack of aqueous solubility, respectively. Cell permeability is higher for lipophilic inhibitors and inversely related to their size (Knight & Shokat, 2005).

HC-3, a hydrophilic compound, was impermeable to the cell (Okuda *et al*., 2002). Similarly, CK37, despite being uncharged, did not inhibit trophozoites in vivo, possibly due to its size and lipophilicity, which may hinder transport across the plasma membrane or lack charge interactions at the hydrophobic choline binding site (Clem *et al*., 2011; Trousil *et al*., 2016).

The study's results demonstrated that flavopiridol induced cell lysis at micromolar concentrations, reducing trophozoite viability by approximately 50% at 3.9 µM. HDTAB and H-89 were also found to induce cell death. Interestingly, these three compounds exhibited lower values compared to their IC_{50}, potentially due to substrate phosphorylation and other cellular signaling pathway outputs. The higher cellular activity observed may suggest that these inhibitors target other cellular components besides EhCK (Knight & Shokat, 2005). HDTAB has been shown to inhibit choline kinase in other parasites like *P. falciparum* (Choubey *et al*., 2007) and *Theileria equi* (Gopalakrishnan *et al*., 2016), but the mechanism of cell lysis induction in *E. histolytica* remains unclear.

HDTAB is known to inhibit ATP synthase in human tumor mitochondria (Ito *et al*., 2009). However, since *E. histolytica* possesses mitosomes instead of mitochondria, which rely on cytosolic ATP production through glycolysis (Saavedra *et al*., 2005), the inhibition mechanism in *E. histolytica* may differ. Further analysis on substrate incorporation and phosphocholine production is needed to validate HDTAB's mechanism of action.

When trophozoites were incubated with H-89, an ATP competitive inhibitor, cell lysis occurred around 97 µM. The bioavailability of a compound depends on its diffusion rate down the concentration gradient across the cell membrane and its rate of efflux by pumps (Knight & Shokat, 2005). *E. histolytica* P-glycoproteins (EhPgps) found in the plasma membrane play a role in avoiding drug accumulation in trophozoites. EhPgps regulate drug accumulation by either actively pumping the drug out of the plasma membrane or concentrating it within vesicles before releasing it from the membrane (Bañuelos *et al*., 2002). The longer preincubation time for H-89 might have been necessary to reach steady state before stimulating the trophozoites (Knight & Shokat, 2005).

RNA interference (RNAi) allows for specific and effective inhibition of gene expression without affecting genomic DNA. This technique has been widely applied to eukaryotic organisms since its first observation in *C. elegans* (Fire *et al*., 1998) and has been successful in *E. histolytica* using various RNA variants such as synthetic siRNA duplex, long dsRNA, dsRNA, and shRNA (Zhang *et al*., 2011). Soaking siRNA into trophozoites via the permeable cell membrane has proven effective in silencing endogenous genes (Ocádiz-Ruiz *et al*., 2013). The most notable study by Vayssié *et al*. (2004) reported a 90% decrease in γ-tubulin protein and 51% decrease in mRNA using synthetic duplex siRNA. These results demonstrate the potential for direct siRNA delivery to effectively and specifically silence *E. histolytica* gene expression.

The identification of potent inhibitors and siRNA targeting *E. histolytica* choline kinase (EhCK) represents a crucial step towards the development of efficacious and safe antiamoebic agents. Given the conserved nature of critical domains within the active site of choline kinases, the design of selective inhibitors for EhCK has been challenging. In this study, potential inhibitors and siRNA were identified to selectively target EhCK while avoiding inhibition of the human homologue, hCKα.

One of the study's limitations was the restricted number of chemical compounds tested. To enhance future investigations, it is suggested to explore additional CK choline competitive inhibitors, such as MN58b, V-11-0711, and RSM-932A. Additionally, investigating the combined effects of two inhibitors that target both choline and ATP binding sites of the choline kinase would be intriguing. Another aspect that warrants improvement is the evaluation of the knockdown of SiRNA on the assessment of protein levels post gene silencing. Unfortunately, the unavailability of specific primary antibodies for *E. histolytica* EhCK hindered this measurement. For forthcoming experiments, it is advisable to monitor growth and collect *E. histolytica* cell lysate after RNAi. Protein expression can be gauged using antibodies that react with similar proteins, potentially capable of binding to *E. histolytica*.

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Conflict of interest statement

The author declares that they have no conflict of interests.

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