In Vitro Testing of Potential *Entamoeba histolytica* Pyruvate Phosphate Dikinase Inhibitors

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Abstract. Adverse effects and resistance to metronidazole have motivated the search for new antiamoebic agents against *Entamoeba histolytica*. Control of amoeba growth may be achieved by inhibiting the function of the glycolytic enzyme and pyruvate phosphate dikinase (PPDK). In this study, we screened 10 compounds using an in vitro PPDK enzyme assay. These compounds were selected from a virtual screening of compounds in the National Cancer Institute database. The antiamoebic activity of the selected compounds was also evaluated by determining minimal inhibitory concentrations (MICs) and IC₅₀ values using the nitro-blue tetrazolium reduction assay. Seven of the 10 compounds showed inhibitory activities against the adenosine triphosphate (ATP)/inorganic phosphate binding site of the ATP-grasp domain. Two compounds, NSC349156 (pancratistatin) and NSC228137 (7-ethoxy-4-[4-methylphenyl] sulfonyl-3-oxido-2, 1, 3-benzoxadiazol-3-ium), exhibited inhibitory effects on the growth of *E. histolytica* trophozoites with MIC values of 25 and 50 µM, and IC₅₀ values of 14 and 20.7 µM, respectively.

INTRODUCTION

Amoebiasis, caused by the protozoan parasite, *Entamoeba histolytica*, is the third most reported cause of mortality from parasitic diseases and has the greatest impact on populations in developing countries.¹ Almost 50 million people are infected with this parasite, resulting in 40,000–100,000 deaths annually in tropical and subtropical countries, particularly in Asia, Africa, and South America.² The transmission of *E. histolytica* cysts is by the fecal–oral route from contaminated water or food. Trophozoites of this parasite are able to invade the intestinal mucosa causing dysentery, fever, and abdominal pain. The trophozoites may spread to other organs, such as liver and lungs, causing abscesses and, in severe cases, death.^{3,4}

The infection is primarily treated using an antiamoebic therapy. Metronidazole is the most widely used drug to combat luminal and hepatic amoebiasis, but it is toxic and may be mutagenic if administered at high doses or used for long-term treatment.⁵ It has a metallic taste but is usually well tolerated, although it may cause nausea, vomiting, and abdominal cramps.⁶ Moreover, studies have shown that differences in drug sensitivity among *E. histolytica* isolate, indicating that there might be a small percentage of amoebae that are either resistant to metronidazole or may eventually become resistant to the drug owing to its indiscriminate use.^{7–11} Furthermore, metronidazole can cross the placental barrier, thus limiting its use in pregnant women.¹² Therefore, new antiamoebic agents are urgently required to overcome the limitations of these treatments.

Glycolysis is an important pathway for adenosine triphosphate (ATP) supply and provides carbon skeleton precursors for the synthesis of macromolecules. It is the main metabolic pathway in *E. histolytica* because this amoeba lacks the genes that encode the enzymes of the Krebs cycle and oxidative phosphorylation; consequently, it relies on substrate-level phosphorylation to provide high-energy compounds.^{13,14} This protozoan parasite uses an unusual PPI-dependent glycolytic pathway in which ATP-dependent phosphofructokinase is replaced by PPI-dependent phosphofructokinase

(PPI-PFK), and pyruvate kinase is replaced by a PPI-dependent pyruvate phosphate dikinase (PPI-PPDK).^{13,15} A strategy to develop new drugs to kill the parasite has been proposed that exploits differences between parasite and human glycolytic pathway enzymes.^{16–18} PPI-PFK and PPDK have been proposed as therapeutic targets for drug design against *E. histolytica* because of their absence in human cells.^{19,20} Saavedra et al.¹³ reported that PPDK may exert greater control of glycolytic flux in *E. histolytica* than PPI-PFK. PPDK catalyzes the reversible conversion of phosphoenolpyruvate (PEP), adenosine monophosphate (AMP), and inorganic phosphate (Pi) to pyruvate and ATP in the presence of Mg²⁺ and NH₄⁺ ion activators. It contains two active sites, located in the N- and C-terminal domains; the N-terminal domain (ATP-grasp domain) carries out the ATP/Pi reaction, and the C-terminal domain carries out the PEP/pyruvate reaction. Site-directed mutagenesis studies of the catalytic site within the ATP-grasp domain of *Clostridium symbiosum* PPDK revealed that mutation of K22, R92, D321, E323, and Q335 resulted in an inactive PPDK, indicating it to be a target for enzyme inhibition.²¹

In the present study, results from molecular modeling and virtual screening of more than 140,000 compounds in the National Cancer Institute (NCI) database against the ATP/Pi binding site of PPDK were used. The top 10 ranking compounds were selected for preliminary investigations into their inhibitory actions against *E. histolytica* PPDK. Subsequently, in vitro antiamoebic activity of potential compounds was performed against the HM-1: IMSS strain of *E. histolytica* using the microdilution method.

MATERIALS AND METHODS

Structural-based modeling of recombinant PPDK and virtual screening of potential inhibitors. Protein modeling and virtual screening of inhibitors against PPDK was outsourced to Aexmoreprima Sdn Bhd (Malaysia). Briefly, a three-dimensional (3D) structure of PPDK was produced by a comparative modeling approach using the MODELLER software (<https://salilab.org/modeller/>) and validated using the PROCHECK software (<http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/>). Potential binding sites in the PPDK structure were detected according to a site-directed mutagenesis study of the ATP-binding site.²¹ The proposed active site residues were K22, R92, R135, D280, D321, E323, Q335,

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and R337. Drugs that potentially target PPDk were identified using the NCI database and AutoDock software (<http://autodock.scripps.edu/>). The virtual NCI screening results were then sorted based on binding energy. Each of top 100 compounds from the initial screen was manually assessed to select those that would favorably interact with the following important binding site residues: K21, R91, D323, E325, Q337, and R339. The PPDk binding sites are composed of charged residues, three of which are positively charged (K21, R91, R339); therefore, hydrogen bond and π -cation interactions were used as determinants to select potential hit compounds.

Preparation of stock solutions. Metronidazole and other compounds were obtained as pure salts from Sigma-Aldrich (St. Louis, MO) and the NCI, Rockville, MD, respectively. Metronidazole and NCI compound stock solutions were prepared at 10 mM in dimethyl sulfoxide (DMSO) and stored at -20°C until use. The starting concentration of metronidazole and NCI compounds was 200 μM , and dilutions were made in the same medium.

Parasite and culture conditions. All experiments were carried out using *E. histolytica* strain HM-1: IMSS, which was obtained from the School of Health Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia. The cells were cultured axenically using Diamond's TYI-S-33 medium, supplemented with 12.5% heat inactivated bovine serum with 80% filled medium in screw-capped tubes at 36°C . The medium was changed every 48–72 hours. Subculturing was routinely performed every 48 hours by replacing the old medium with 10 mL fresh medium without detaching the monolayer in Nunc slant-end culture tubes. Cell viability was determined using microscopy and a trypan blue dye exclusion test.

Expression and purification of recombinant protein. The protocol from Saidin et al.²² was performed to express and purify recombinant PPDk (rPPDK). The expression of rPPDK was detected by western blotting using a mouse anti-His.tag-HRP monoclonal antibody (Novagen, Madison, WI) at 1:5,000 dilution. The purity of rPPDK was analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the protein concentration was determined using the Bio-Rad Protein assay (Bio-Rad, Hercules, CA).

rPPDK enzyme assay. The rate of reaction catalyzed by rPPDK was determined in the direction of ATP and pyruvate synthesis via the coupling lactate dehydrogenase (LDH) (Sigma-Aldrich) and following the oxidation of NADH at 340 nm. The assay conditions were based on a previous report with slight modification.²³ The reaction mixture contained 50 mM imidazole, pH 6.3, 20 mM NH_4Cl , 4.5 mM MgCl_2 , 0.2 mM PEP, 0.2 mM AMP, 1.25 mM PPi , 0.2 mM NADH, and 4 U of LDH in a 200- μL reaction volume. The reaction was started by adding 5 μg of rPPDK. Michaelis-Menten constant (K_m) values for PEP, PPi , and AMP were determined using GraphPad software (GraphPad, La Jolla, CA).

Bioactivity analysis of NCI compounds as potential inhibitors against rPPDK. Previously, active site predictor software had suggested the ATP/ Pi site as the best target to inhibit PPDk activity. Thus, in this study the selected NCI compounds compete with the substrate (AMP) to bind to the PPDk active site. For initial inhibitor screening, the test was carried out in a 200- μL assay. The prepared solutions contained varying AMP concentrations (0.5-fold to 8-fold of the AMP K_m) with saturating concentrations of cosubstrates (0.2 mM PEP and 1.25 mM PPi) and metal ion cofactors

(4.5 mM MgCl_2 and 20 mM NH_4Cl) in 50 mM imidazole (pH 6.3, 30°C). The concentrations of inhibitors tested in the assay solution were 5, 10, 15, and 20 μM , depending on compound availability. In each case, the reaction was initiated by the addition of rPPDK. The inhibition constants (K_i) for the inhibitors were determined from the initial velocity data obtained at varying AMP concentrations and fixed-saturating concentrations of cosubstrates and cofactors. The kinetic data were analyzed by GraphPad Prism software to determine the K_i .

In vitro screening of selected compounds against *E. histolytica* trophozoites by a minimum inhibitory concentration (MIC) assay. Trophozoites in a flask were chilled on ice for 5 minutes to detach them from the surface of the flask. Then, the medium containing trophozoites was transferred to a 15-mL tube and centrifuged at $440 \times g$ for 1 minute. The supernatant was discarded and 4 mL of fresh medium was added to the pellet. After mixing, 50 μL of cell suspension were mixed with an equal volume of 0.4% trypan blue stain, and cells were counted under a microscope using a Neubauer hemocytometer. Approximately, 1×10^5 cells/mL in the TYI-S-33 medium were seeded into selected wells, in rows A to H, of a 96-well flat-bottom microtiter plate (Nalgene Nunc, Rochester, NY). For the metronidazole positive control assay, columns 6–8 were used, whereas columns 9–11 were used for the compound assay. Columns 1–2, 3–5, and 12 were used as control wells, containing TYI-S-33 medium with trophozoites, TYI-S-33 medium with 0.5% DMSO and trophozoites, and TYI-S-33 medium only, respectively. The volume of each well was adjusted to 300 μL (about 80% of well capacity) by adding fresh TYI-S-33 medium to each well, except for wells in column 12. The plate was incubated overnight at 36°C under anaerobic conditions. When the trophozoites in all wells were 100% confluent, compounds and metronidazole were added to the appropriate wells. The final compound concentrations in rows A to H were 100, 50, 25, 12.5, 6.3, 3.1, 1.6, and 0.8 (μM), respectively. One hundred and fifty microliters of medium was discarded from wells containing trophozoites and replaced with the same volume of various concentrations of compounds or metronidazole at 1:1 dilutions. Trophozoite growth in each well was monitored microscopically for motility and morphological changes (rounding-up) after 24 and 48 hours of incubation. Comparisons were made between the positive control (metronidazole-treated cells) and test wells using an inverted microscope. All tests were performed in triplicate and were repeated three times. Trophozoite growth was scored according to Table 1, and minimal inhibitory concentrations (MICs) was defined as the lowest concentration of compound/metronidazole dilution at which a 1+ score was obtained in the majority (90%) of the triplicate wells.

TABLE 1
Scoring of trophozoites growth according to Upcroft and Upcroft⁶

Score	Descriptions
1+	Dead or significantly fewer (not > 20% coverage of well surface) and 90% rounded up than the control well
2+	20–50% coverage of the well surface and some motility
3+	An almost confluent well (50% coverage of the well surface) and much motility
4+	A confluent well (100% coverage of the well surface)

TABLE 2

Virtual screening results of NCI diversity set sorted based on compounds interacting with important binding site residues of pyruvate phosphate dikinase *Entamoeba histolytica*

NCI code	Compound structure	Molecular weight, g/mol	Molecular formula	XlogP3	Hydrogen bond	Pi-cation	Lowest binding energy
NSC202386		521.5	C ₂₇ H ₁₉ N ₇ O ₅	4.7	S241, K21, V243, R91, M242	–	–9.4
NSC227186		697.1	C ₃₅ H ₃₇ ClN ₂ O ₁₁	5.3	V243, G100, T104, R91	R91	–12.9
NSC228137		334.4	C ₁₅ H ₁₄ N ₂ O ₅ S	2.9	R91, T104	Q337	–8.8
NSC228150		303.3	C ₁₃ H ₉ N ₃ O ₄ S	2.2	R339, G100, K21	–	–8.7
NSC270071		391.5	C ₁₆ H ₁₇ N ₅ O ₃ S ₂	1.5	R91, T104	–	–8.5
NSC329065		369.5	C ₂₀ H ₂₃ N ₃ O ₂ S	3.8	R91, T104	–	–9.0

(continued)

TABLE 2
Continued

NCI code	Compound structure	Molecular weight, g/mol	Molecular formula	XlogP3	Hydrogen bond	Pi-cation	Lowest binding energy
NSC339594		339.4	C ₁₆ H ₁₃ N ₅ O ₂ S	3.3	R339, G100	R91	-8.5
NSC349156		325.3	C ₁₄ H ₁₅ NO ₈	-1.6	R91, T104, R339, M102, G278, G100, K21	-	-8.0
NSC379696		499.4	C ₁₉ H ₁₆ Cl ₂ N ₄ O ₄ S ₂	4.8	R91, T104, R339	Q337	-10.2
NSC380802		363.4	C ₁₉ H ₁₇ N ₅ O ₃	3.8	R91, K21, M102, T104	-	-9.1

This table appears in color at www.ajtmh.org.

In vitro cell viability assay to determine IC₅₀ value. The sensitivity of *E. histolytica* to compounds was tested by the nitro blue tetrazolium (NBT) reduction method. Each of the selected compounds and metronidazole was tested in triplicate. *E. histolytica* trophozoites were harvested from 24 hour-old cultures and suspended in the medium. The trophozoite count was adjusted to 1×10^5 trophozoites/mL, and the assay was carried out in wells of microtiter plates. Briefly, 150 μ L of drug was added to row A of the microtiter plate. Meanwhile, the medium was added to rows B to H. Then, serial dilutions of

the drug were performed with drug concentrations in rows A to H at 100, 50, 25, 12.5, 6.3, 3.1, 1.6, and 0.8 (μ M), respectively. Then, 150 μ L of trophozoite suspension (1×10^5 cells/mL) was added to all rows (A to H). Each test included a control (without drug) and blank wells (medium only). The plates were incubated at 37°C for 24 hours. The medium was then discarded and wells washed with prewarmed Hank's balanced salt solution (HBSS pH 7.2) (Gibco, Waltham, MA). Thereafter, 100 μ L of NBT in HBSS was added to each well and plates incubated at 37°C for 45 minutes. The medium was then aspirated, wells

FIGURE 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of recombinant PPDK (rPPDK) purification. Lane M: Precision Plus Protein™ Unstained Standard Marker (Bio-Rad); Lane 1: Flow-through supernatant; Lanes 2–4: washing steps using 20, 30, and 40 mM imidazole, respectively; Lanes 5–8: purified rPPDK eluted with 250 mM imidazole.

washed twice with HBSS and 200 μ L DMSO (100% v/v) added to each well. Following incubation at 37°C for 10 minutes, the optical density (OD₅₄₀) was measured with an ELISA spectrophotometer. The percentage of nonviable organisms, which failed to metabolize NBT and, therefore, did not produce the dark blue formazan product, was determined by the formula:

Percentage of nonviable organisms at each drug concentration

$$= 100 - \left(\frac{\text{Test OD}_{540} - \text{Blank OD}_{540}}{\text{Control OD}_{540} - \text{Blank OD}_{540}} \right) \times 100$$

The IC₅₀ concentration of each compound was determined using GraphPad Prism software at a 95% confidence level.

The mortality rate of trophozoites was evaluated at IC₅₀ values to assess the efficiency of compounds at killing the cells. Approximately 1×10^5 trophozoites/mL in 300- μ L TYI-S-33 medium were seeded into row B wells (columns 1–12) of a 96-well flat-bottom microtiter plate. The plate was incubated overnight at 36°C under anaerobic conditions. When trophozoites in all wells were 100% confluent, treatments with compounds and metronidazole were performed. One hundred and fifty microliters of medium was discarded from wells containing trophozoites and replaced in columns 4–6 with the same volume of metronidazole at the concentration of its IC₅₀ value, and in columns 7–9 with compounds at their IC₅₀ value concentrations, while 150- μ L medium containing 0.5% DMSO was placed into wells in columns 1–3. Wells in columns 10–12 received 150- μ L medium only. The trophozoites were incubated for 12, 24, and 48 hours at 36°C and cell viability was determined as described previously. The mortality rate of trophozoites was calculated for each time tested according to the formula:

$$\text{Mortality rate}(\%) = \frac{\text{Number of viable cells(DMSO)} - \text{Number of viable cells(assay)}}{\text{Number of viable cells(DMSO)}} \times 100$$

The mortality rates of *E. histolytica* in response to tested compounds were compared with the corresponding mortality

FIGURE 2. Western blot analysis of recombinant pyruvate phosphate dikinase using anti-His-HRP. Lane M: Precision Plus Protein™ Unstained Standard Marker (Bio-Rad); Lane 1: *Escherichia coli* whole cells transformed with pET28(+) vector; Lane 2: *E. coli* whole cell expressed recombinant PPDK.

rates of the reference drug, metronidazole. The *P* value was calculated using Student's *t* test.

RESULTS

Modeling of recombinant PPDK and virtual screening of potential inhibitors. We modeled *E. histolytica* PPDK using the MODELLER software and validated it by using the PROCHECK software. The latter generated a Ramachandran Plot which showed that the modeled protein was reliable with 92.2% residues in the most favored regions, 7.7% in additional allowed regions, no residues in disallowed regions, and 0.1% in generously allowed regions. Virtual screening of compounds from the NCI database using AutoDock software identified 10 potential inhibitors; this was based on their interaction with important binding site residues in the ATP-grasp domain active site of *E. histolytica* PPDK (Table 2).

Expression and purification of rPPDK. The size of the rPPDK protein (~98 kDa) was consistent with its expected molecular mass (Figure 1), as confirmed by western blotting using an anti-His-HRP antibody (Figure 2). Approximately, 1.5 mg soluble rPPDK per liter of culture was obtained.

rPPDK enzyme assay and kinetic data analysis. The optimal pH and temperature for the rPPDK enzyme assay were determined to be 6.3 and 30°C, respectively. The specific activity of purified rPPDK was 16.5–23.1 U/mg. The rPPDK activity was analyzed without removing the 6-His tag, and kinetic parameters revealed hyperbolic dependencies on all the substrates, which was in agreement with Michaelis–Menten kinetics. For each of the three substrates involved in the forward (pyruvate production) reactions, the apparent *K_m* values were 39.4 μ M for PEP, 7.8 μ M for AMP, and 90.8 μ M for PP_i.

TABLE 3
Comparison of K_m for three substrates of pyruvate phosphate dikinase (PPDK) from different sources

Source	PEP (μ M)	PPI (μ M)	AMP (μ M)	Reference
<i>Entamoeba histolytica</i> recombinant PPDK (rPPDK)	39.4	90.8	7.8	This work
<i>E. histolytica</i> rPPDK	21	100	< 5	Saavedra et al. ²³
<i>E. histolytica</i> PPDK	70	100	Not determined	Reeves ⁴¹
<i>Giardia lamblia</i>	24	29	5	Hrdy et al. ⁴²
<i>Bacteroides symbiosus</i>	60	100	3.4	South and Reeves ⁴³
Maize	140	40	10	Edwards et al. ⁴⁴
<i>Trypanosoma brucei</i>	40	50	7.5	Bringaud et al. ²⁹
<i>Thermoproteus tenax</i>	500	80	20	Tjaden et al. ⁴⁵
<i>Microbispora subsp. aerata</i>	280	38	5	Eisaki et al. ⁴⁶
<i>G. lamblia</i>	33.3	29	1.3	Hiltbold et al. ⁴⁷
<i>Propionibacterium shermanii</i>	36	120	15	Cooper and Kornberg ²⁸
<i>Acetobacter xylinum</i>	100	67	1.6	Cooper and Kornberg ²⁸

Bold text indicates data from the present study.

For comparison, K_m values of PPDK from other organisms for the three substrates are shown in Table 3.

Inhibition studies. The biological inhibition study was performed on the 10 compounds that were identified as potential rPPDK inhibitors (Table 2). The assay solutions contained 3.9–64 μ M AMP (range 0.5- to 8-fold K_m). Activity data are shown in Table 4. The kinetic assay showed seven compounds that exhibited competitive inhibition against rPPDK with K_i in the range of 3.8–34.6 μ M. In vitro screening of potential inhibitors showed that the compound with the lowest inhibition constant was NSC228137 (K_i of 3.8 μ M). This was followed by NSC270071, NSC227186, NSC329065, NSC349156, NSC379696, NSC380802, NSC339594, NSC228150, and NSC202386. Three compounds, namely NSC202386, NSC228150, and NSC339594, showed a K_i value greater than the inhibitor concentration (> 20 μ M), indicating no inhibition.

Minimal inhibitory assay of NCI compounds against *E. histolytica* trophozoites. Antiamoebic activity of NCI compounds was assessed using a broth dilution method with metronidazole as the reference. Trophozoites were monitored for motility and morphological changes and scored accordingly. Table 5 summarizes the antiamoebic activity of the potential compounds and the control drug. In our preliminary study, the MIC of metronidazole was 12.5 and 6.3 μ M after incubation for 24 and 48 hours, respectively. Two compounds, NSC228137 and NSC349156, exhibited potent antiamoebic activity against *E. histolytica*. NSC349156 showed the most potent activity with MIC values of 25 μ M after 24 hours and 12.5 μ M after 48 hours. Meanwhile, NSC228137 exhibited moderate activity against *E. histolytica* with a MIC value of 50 μ M after incubation for 24 hours and a similar value after

48 hours incubation (MIC = 50 μ M). The remaining eight compounds showed no inhibition after 48 hours of treatment; the morphology of trophozoites did not change and the cells were 100% confluent. This suggested that these compounds were ineffective against *E. histolytica*. Based on these results, NSC349156 (pancratistatin) and NSC228137 (7-ethoxy-4-(4-methylphenyl) sulfonyl-3-oxido-2, 1, 3-benzoxadiazol-3-ium) were used for further in vitro antiamoebic activity assays.

Cell viability assay of anti-amoebic compounds. The IC_{50} values obtained from the absorbance readings and represented in a nonlinear dose response curve derived from GraphPrism are presented in Table 6. In the presence of NSC349156 and NSC228137, the growth inhibition and mortality of *E. histolytica* increased in a concentration dependent manner. NSC349156 exhibited the highest antiamoebic activity with an IC_{50} of 14 μ M after 24 hours of incubation and 5.8 μ M after 48 hours of incubation. Meanwhile, NSC228137 showed lower antiamoebic activity compared with NSC349156, with IC_{50} values of 20.7 and 14.5 μ M after incubation for 24 and 48 hours, respectively. It was noted that when compared with metronidazole (IC_{50} = 6.2 μ M after incubation for 24 hours), NSC349156 showed lower antiamoebic activity at 24 hours, but similar activity at 48 hours (5 μ M).

The mortality rate of trophozoites was evaluated at the IC_{50} values of the selected compounds (NSC228137 and NSC349156). Treatment of trophozoites with 20.7 μ M of NSC228137 and 14 μ M of NSC349156 caused different mortality rates depending on the period of incubation, as shown in Table 7. Of the two compounds, NSC349156 showed greater inhibition against *E. histolytica*, with a 75.7% mortality rate after treatment of 24 hours. This inhibition was not significantly different from that exhibited by metronidazole

TABLE 4
Inhibition constants value (K_i) of various compounds against recombinant PPDK

Compound ID	Inhibition constant, K_i (μ M)	Mode of inhibition	Michaelis Manten constant, K_m (μ M)	Maximum rate of reaction, V_{max} (U/mg)
NSC202386	34.6	Competitive inhibition	7.3	4.5
NSC227186	11.3	Competitive inhibition	46.6	18.8
NSC228137	3.8	Competitive inhibition	2.2	5.2
NSC228150	27.6	Competitive inhibition	27.6	9.3
NSC270071	14.8	Competitive inhibition	12.4	7.1
NSC329065	11.5	Competitive inhibition	23.3	10.2
NSC339594	22.4	Competitive inhibition	45.9	15.2
NSC349156	15.9	Competitive inhibition	62.8	14.8
NSC379696	16.6	Competitive inhibition	55.7	14.9
NSC380802	16.6	Competitive inhibition	23.6	6.8

TABLE 5

Minimal inhibitory concentration values for top 10 compounds and metronidazole treated after 24 and 48 hours of treatment

Compound ID	MIC assay (μM)	
	24 hours	48 hours
NSC202386	> 100	> 100
NSC227186	> 100	> 100
NSC228137	50	50
NSC228150	> 100	> 100
NSC270071	> 100	> 100
NSC329065	> 100	> 100
NSC339594	> 100	> 100
NSC349156	25	12.5
NSC379696	> 100	> 100
NSC380802	> 100	> 100
Metronidazole	12.5	6.3

(80.5% mortality rate). The *t* test analysis of the mortality rates of NSC228137 compared with those of metronidazole showed statistically significant differences ($P < 0.05$) at all treatment time points. After 48 hours of treatment with 20.7 μM NSC228137, only 56.9% of the trophozoites died.

Furthermore, the death of trophozoites was reflected by cell rounding and cell lysis. The morphological structures of untreated (control) and treated, as well as DMSO-treated cells are shown in Figure 3. The untreated cells exhibited the distinct morphology of pseudopodia. Meanwhile, the treated *E. histolytica* rounded-up owing to the shortening and loss of their pseudopodia, which eventually led to their detachment from the surface of the well and caused them to float in the culture medium. Moreover, treatment of trophozoites with 0.5% DMSO did not remarkably affect cell growth as the cells remained metabolically active and viable.

DISCUSSION

Metronidazole and tinidazole are the most commonly used drugs to treat amoebiasis.²⁴ Other nonimidazole drugs, such as nitazoxanide, paromomycin, and niridazole, are less effective against the cyst stage of *E. histolytica*.²⁵ Resistance of *E. histolytica* to metronidazole, as well as variability of efficacy among strains of amoebae, and relapse of intestinal and extraintestinal amoebiasis have been reported.^{8,9,26,27}

PPDK is not found in humans and is one of the key enzymes in the *E. histolytica* glycolytic pathway; therefore, it is a potential novel target for the development of new anti-amoebic agents.^{21,23,28} The present report describes results of in silico screening of a chemical compound library against *E. histolytica* PPDK and preliminary data of the effect of selected compounds on the proliferation of *E. histolytica*. The NCI database was used in the virtual screening because it

TABLE 6

IC₅₀ values of NSC349156, NSC228137, and metronidazole on the growth of *Entamoeba histolytica* trophozoites at different incubation periods

Compounds	IC ₅₀ value (μM)	
	24 hours	48 hours
NSC228137	20.7	14.5
NSC349156	14	5.8
Metronidazole	6.2	5

TABLE 7

The effect of compounds and metronidazole at IC₅₀ on the in vitro growth of *Entamoeba histolytica* trophozoites at different incubation periods

Incubation time (hour)	Mortality rate (%)		
	12	24	48
NSC228137	17.3*	37.6*	56.9*
NSC349156	29.7*	75.7†	89.1†
Metronidazole	34.8	80.5	91.4

* Student's *t* test [$P < 0.05$; significantly different].

† Student's *t* test [$P > 0.05$; not significantly different].

contains compounds with rich structural and pharmacophore diversity. Although this database is usually used for cancer research, its diversity makes it useful to search for potential scaffolds for use in anti-amoebic drug discovery. The NCI virtual screening results were sorted based on binding energy and were manually verified to select compounds that showed favorable interaction with important residues at the binding site of the ATP/Pi active site.

The rPPDK enzyme in this study proved to be catalytically active and exhibited kinetic parameters comparable with the rPPDK produced by Saveedra et al.²³ In addition, the kinetic properties were similar to PPDK of *P. shermanii*²⁸ and *Trypanosoma brucei*.²⁹ The selected compounds were then tested for their ability to block rPPDK activity. NSC228137 was the most potent inhibitor, showing the lowest *K_i* value of 3.8 μM . The compound increased the *K_m* value of the enzyme, suggesting that it binds competitively with the substrate at the active site. MIC assays identified two compounds that inhibit *E. histolytica* growth: NSC228137 and NSC349156. Surprisingly, eight compounds showed no cytotoxic effect against *E. histolytica* although they showed inhibitory effects in the enzymatic assay. A possible explanation for this is that the compounds may exhibit inadequate bioavailability; i.e., they are not able to efficiently target cells. Poor bioavailability is linked with low solubility. Good inhibitors usually have a few polar groups and are often most "comfortable" in the mostly hydrophobic environment of an enzyme active site.^{30,31} Hence, increasing the number of polar groups on the ligand will often improve its solubility by two orders of magnitude or more. In addition, membrane permeability can greatly affect the biological activity of some compounds for intracellular targets. Thus, these compounds may have been very active on cell-free enzymes, but their membrane permeability may be low, eliminating their activity in the cell.^{32,33} Structural and chemical modification of the compounds identified in this study may be able to increase their potency and bioavailability. In addition, high throughput screening might be useful to screen large compound libraries to discover more potent compounds.

The results of the anti-amoebic activity showed that the IC₅₀ value of NSC349156 was lower than that of NSC228137, whereas the mortality rate of *E. histolytica* after 24 hours treatment with NSC349156 at the IC₅₀ value was 75.7%, slightly lower than that of metronidazole. Thus, NSC349156 is a good candidate for the development of new anti-amoebic agents, even though the potency of the compound was modest.

The strategy used in this study has been widely applied by others for in silico screening of compound libraries for binding affinity to target receptors.^{25,34–36} Furthermore, in addition to

FIGURE 3. Microscopic examination of *Entamoeba histolytica* at 24 hours (A) Untreated cells show extended pseudopodia from the ectoplasm (black arrow); (B) *E. histolytica* treated cells with 0.5% DMSO; (C) *E. histolytica* treated cells with metronidazole show rounding up of cells. Rounding-up refers to the ball-like *Entamoeba* trophozoite with no evidence of purposeful movement and no pseudopodia; (D) *E. histolytica* treated cells with NSC349156 show rounding up of cells; and (E) *E. histolytica* treated cells with NSC228137. Magnification $\times 100$. This figure appears in color at www.ajtmh.org.

screening binding energy, manual verification was used to select compounds that interact with important residues at the binding site of the rPPDK ATP/Pi active site. Chemical and structural modification can be performed in the future to enhance the potency and therapeutic index of NSC349156.

NSC349156 is known as pancratistatin (PST-1). It is an essential metabolite that has therapeutic benefits and can be found in *Pancreatum maritimum* L. This compound and other similar alkaloids also exhibit antimalarial, antiviral, antineoplastic, and antiparasitic (against *Encephalitozoon intestinalis*) activity.^{37,38} In addition, pancratistatin that was extracted and purified from the *Pancreatum littorale* inhibited *E. intestinalis* infection without affecting host cells.³⁹ Another study conducted by El-Sayed et al.³⁸ found pancratistatin to be responsible for the cysticidal activity of *P. maritimum* against *Achantamoeba castellanii*. Pancratistatin at very low concentrations ($< 1 \mu\text{M}$) also induced apoptosis, specifically in a broad range of cancer cell lines. Interestingly, pancratistatin

did not have any toxic effects on noncancerous cells, such as primary human endothelial cells or normal human fibroblasts.³⁷ These results suggest that pancratistatin specifically targets rPPDK. Gabrielsen et al.⁴⁰ reported that *Japanese encephalitis* virus-infected mice showed 100% survival over 28 days when treated with a high dose of 2% ethanolic-saline solution of pancratistatin (6 mg/kg/day). In another study, no cytopathic effect on host cells was reported when 5 μM pancratistatin was used.³⁷ Based on these observations and the results obtained in this study, we postulate that this compound is nontoxic at the concentration that is active against *E. histolytica*. However, the compound requires testing in human cell lines and in in vivo toxicity studies to determine whether it has promise for treating amoebiasis.

In conclusion, this study identified potential inhibitors that blocked the growth of the protozoan pathogen, *E. histolytica*. Two NCI compounds showed potential for development as new antiamoebic agents, especially pancratistatin. NSC228137 also

shows promise as an alternative antiamoebic agent and thus merits further investigation.

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