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Entamoeba histolytica alcohol dehydrogenase 2 (EhADH2) as a target for anti-amoebic agents

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***Objectives*: The current use of metronidazole as an anti-amoebic agent causes significant side-effects. The purpose of this study was to identify alternative compounds with which to treat amoebiasis.**

***Methods*: We tested the effects of cyclopropyl (CPC) and cyclobutyl (CBC) carbinols on the survival of *Entamoeba histolytica* trophozoites and on the enzymatic activities of *E. histolytica* alcohol dehydrogenase 2 (EhADH2), a crucial enzyme in the amoebic fermentation pathway.**

***Results*: At 72 h, the estimated 50% inhibitory concentrations of CPC and CBC were 38.9 and 11.2 μM, respectively. The EhADH2 alcohol and aldehyde dehydrogenase activities were inhibited by 1.82 μM CPC and 0.89 μM CBC *in vitro*.**

***Conclusions*: CPC and CBC are expected to be non-toxic to humans at the concentrations required to eliminate *E. histolytica* trophozoites. Similarities between EhADH2 and the *Giardia lamblia* AdhE enzyme indicate that CPC and CBC could be effective drugs for treatment of both amoebiasis and giardiasis.**

Keywords: bifunctional proteins, glycolytic pathways, eukaryotic parasites, cycloalkanols, alcohol dehydrogenase E

Introduction

The intestinal protozoan *Entamoeba histolytica* is a major cause of morbidity and mortality worldwide, causing 50 million cases of diarrhoea and 100 000 deaths per year.[1,2] Amoebiasis is primarily treated with the drug metronidazole, even though significant side-effects, such as neurological complications, and the possible selection of a resistant *E. histolytica* strain have been reported.[3–6] These concerns have prompted the search for alternative therapeutic agents. *E. histolytica* lacks mitochondria and obtains its energy from the fermentation of glucose, producing carbon dioxide, acetate and ethanol as end products.[7,8] *E. histolytica* alcohol dehydrogenase 2 (EhADH2), a bifunctional enzyme with both aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) activities, constitutes a key enzyme in this pathway.[7–10] We have previously shown that EhADH2 expression is required for the growth and survival of *E. histolytica* trophozoites,[11] suggesting this enzyme could be a target for new anti-amoebic drugs.

Successful complementation of a mutant *Escherichia coli* strain (SHH31Δ*adhE*) with recombinant EhADH2[12,13] provides an ideal system for studying potential inhibitors of EhADH2. In this study, we show that the cycloalkanol compounds cyclopropyl (CPC) and cyclobutyl (CBC) carbinols inhibit the growth and survival of *E. histolytica* trophozoites. We also demonstrate that these agents specifically affect the ALDH and ADH activities of the recombinant EhADH2.

Materials and methods

Trophozoites of *E. histolytica* strain HM1:1MSS were cultured under axenic conditions in Diamond's TYI-S-33 medium.[11–13] Trophozoites in the log phase of growth were used in all experiments. To determine inhibition of *E. histolytica* growth, standard culture tubes containing an initial inoculum of 5×10^3 trophozoites per tube were grown and counted at 48 and 72 h. Viable trophozoites were counted using a haemocytometer. Growth counts were averaged from three replicate tubes and three separate experiments. Amoebic cultures were closely examined to verify absence of bacterial contamination in tubes. *E. coli* strain SHH31 (Δ*adh zch*::Tn10 *fad*R*met tyr*T)[10,12,13] was used for transformation and expression of the recombinant EhADH2.

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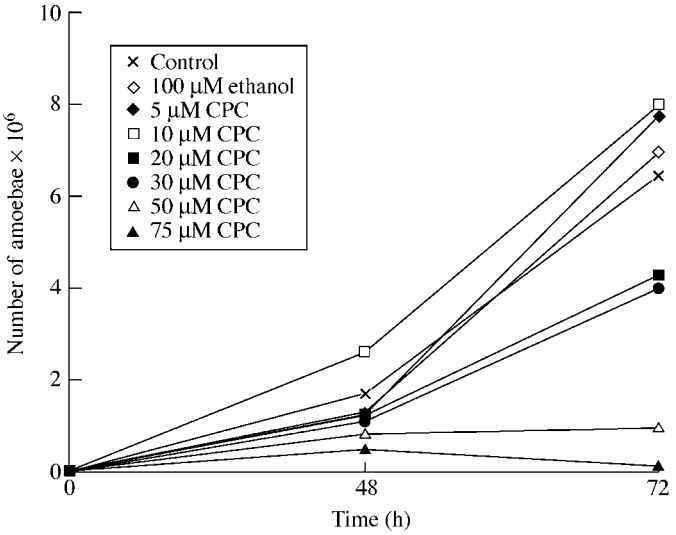

Figure 1. Inhibition of trophozoite growth with CPC. A concentration of 75 μM inhibits trophozoite growth. An initial 5×10^3 HM1:1MSS *E. histolytica* trophozoites were inoculated per tube, and counted at 48 and 72 h.

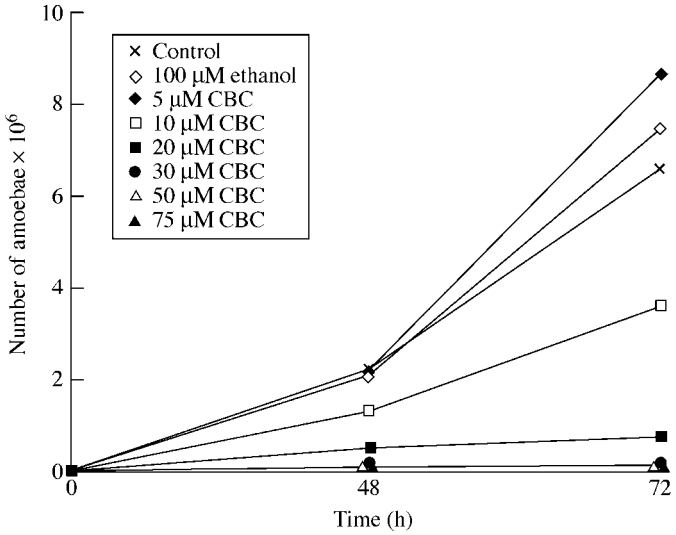

Figure 2. Inhibition of trophozoite growth with CBC. A concentration of 30 μM completely blocks trophozoite growth. An initial 5×10^3 HM1:1MSS *E. histolytica* trophozoites were inoculated per tube, and counted at 48 and 72 h.

For anaerobic growth, the transformed strain was grown on solid (1.5% Bacto Agar; Difco, Sparks, MD, USA) or liquid M9 media[11,13] in anaerobic jars (BBL GasPak system; BBL, Cockeysville, MD, USA) with anaerobic system envelopes (BBL GasPak Plus). Indicator strips were used to confirm anaerobic conditions. A single colony of strain SHH31 (Δ*adhE*) transformed with pEhADH2[11] was grown overnight under aerobic conditions. Bacterial cells were processed as described elsewhere.[10,11,13] Lysate samples were analysed by SDS–PAGE and western blot analyses. Bacterial lysates were used to measure NAD^+-dependent ALDH and ADH activities. ALDH activity was assayed spectrophotometrically by measuring decrease in absorbance at 340 nm, following the oxidation of NADH to NAD using acetyl-CoA as the substrate.[14,15] ADH activity was measured by the same assay, with the substitution of acetaldehyde for acetyl-CoA as the substrate.[14,15] One unit of enzyme activity was defined as that which consumed 1 μmol of NADH or NAD^+/min. Activity values were averaged from three independent samples. Substrate concentrations were based on standard protocols.[13–15]

Results

CPC and CBC inhibited the growth of *E. histolytica* trophozoites (Figures 1 and 2). A CPC concentration of at least 75 μM was required for complete trophozoite growth inhibition (Figure 1; Table 1). In contrast, a CBC concentration of only 30 μM (a 2.5-fold decrease) was required to completely block trophozoite growth (Figure 2; Table 1). The EIC_{50} value for CBC was much lower than that for CPC: 11.2 μM and 38.9 μM, respectively (Table 1). The two compounds also blocked anaerobic growth of wild-type *E. coli* (BL21 DE3) and *E. coli* strain SHH31Δ*adhE* transformed with EhADH2 in plasmid pET3a (data not shown).

CPC and CBC inhibited both enzymatic activities of the EhADH2 protein *in vitro* (Table 2). Assays of ALDH and ADH activities demonstrated a stronger effect of CBC than of CPC. The effects of CPC and CBC on the ALDH activity of EhADH2 were particularly dramatic. At 0.53 μM, CPC decreased the ALDH activity by 53%, while a similar concentration of CBC completely eliminated ALDH activity (Table 2). CBC completely eliminated the ADH activity of EhADH2 at 0.89 μM, while a two-fold increase in concentration (1.82 μM) of CPC was required to obtain similar results (Table 2).

Discussion

EhADH2 is a member of the microbial group III or 'iron activated' alcohol dehydrogenases, with higher homology to prokaryotic than to eukaryotic alcohol dehydrogenases.[16,17] In an attempt to search for specific inhibitors for this enzyme, we tested two alkanols, CPC and CBC. This study shows that both of these carbinols have an inhibitory effect on *in vivo* trophozoite growth (Figures 1 and 2; Table 1) and on *in vitro* EhADH2 enzymatic activities (Table 1). CBC is a stronger inhibitor than CPC, but both compounds affect growth and enzymatic function at micromolar concentrations (Tables 1 and 2). These results suggest that CPC and CBC interfere with trophozoite growth by blocking EhADH2 and, therefore, fermentative metabolism. Our results show an important difference between the drug concentrations required to block parasite growth and to inhibit enzymatic activity *in vitro*. Since EhADH2 is mostly localized in the cytosol, these compounds probably cross the parasite's membrane to affect its metabolism. Although the mechanisms underlying the inhibitory effects of either compound on the anaerobic growth of *E. histolytica* are not known, a likely possibility is that EhADH2 converts both cyclic alcohols into corresponding aldehydes, which then attack the active site residues of the glycolytic enzyme, affecting its activity. This is the mechanism by which unsaturated alcohol analogues, such as allyl alcohol, inhibit ADH enzymes.[18–20] Moreover, in preliminary fluorescence analysis of EhADH2, addition of CBC to a mix of EhADH2 and acetyl-CoA blocked quenching of tryptophans of EhADH2 by the substrate (data not shown). It is possible to speculate that CBC interferes irreversibly with substrate binding of acetyl-CoA.

Table 1. Effect of cycloalkanols CPC and CBC on the growth of *E. histolytica* trophozoites (EIC_{50} at 48 and 72 h are shown)

	No. trophozoites $\times 10^6$ (% inhibition in respect to controls)			
	CPC		CBC	
Concentration	48 h	72 h	48 h	72 h
Control	1.7 (NA)	6.5 (NA)	2.2 (NA)	6.7 (NA)
100 μM ethanol	1.2 (27.1)	7.8 (+19.2)[a]	2.1 (4.5)	7.5 (+12)[a]
5 μM	1.3 (23.5)	7.0 (+7.7)[a]	2.2 (0)	8.7 (+29.8)[a]
10 μM	2.6 (+52.9)[a]	8.0 (+23)[a]	1.3 (41.0)	3.7 (44.8)
11.2 μM[b]	–	–	–	3.2 (50)[c]
12.2 μM[b]	–	–	1.1 (50)[c]	–
20 μM	1.2 (29.4)	4.3 (33.8)	0.5 (77.3)	0.8 (88.1)
30 μM	1.1 (35.3)	4.0 (38.5)	0.1 (95.5)	0.2 (97.0)
38.9 μM[b]	–	3.1 (50)[c]	–	–
50 μM	0.8 (50)	1.0 (84.6)	0.01 (99.5)	0.01 (99.8)
75 μM	0.5 (70.6)	0.1 (98.5)	0.01 (99.5)	0.01 (99.8)

NA, not applicable.
[a]Percentage growth increase, no inhibition.
[b]EIC_{50}, estimated inhibitory concentrations of cycloalkanols required to inhibit 50% amoebic growth.
[c]Estimated no. trophozoites $\times 10^6$ killed by EIC_{50}.

Table 2. *In vitro* inhibition of EhADH2 enzymatic activities (ALDH and ADH) by CPC and CBC

Carbinol (concentration)	ALDH activity (mU/μg)			ADH activity (mU/μg)		
	control	with inhibitor	% inhibition	control	with inhibitor	% inhibition
CPC						
(0.53 μM)	313	149	53	710	473	33
(1.82 μM)	322	0	100	810	0	100
CBC						
(0.53 μM)	326	0	100	796	265	67
(0.89 μM)	307	NA	NA	754	0	100

NA, not applicable.

The different phylogenetic origin and unique structural characteristics of EhADH2 with respect to the human dehydrogenases also suggest that CPC and CBC would bind specifically to this enzyme and probably not to the human ADH and ALDH homologues. We have hypothesized that EhADH2 originated from the fusion of ancestral ADH and ALDH microbial proteins, diverging functionally and structurally from classical unifunctional ADHs and ALDHs.[11] The anaerobic EhADH2 uses iron as the catalytic ion and shares the co-factor binding site for the ADH and ALDH activities.[11] CPC and CBC apparently have strong affinity for this shared binding site. In contrast, humans possess two structurally independent ADH and ALDH aerobic enzymes; ADH is zinc dependent and ALDH requires no catalytic ion. Based on these differences between the amoebic and the human dehydrogenases, we predict little affinity of cycloalkanols for the mammalian enzymes.

Although CPC and CBC can be toxic to humans at millimolar concentrations, our EIC_{50} data indicate that, if administered at micromolar concentrations, these or similar compounds could be used safely as anti-amoebic agents. As anaesthetics, cycloalkanols act on the cytochrome P450 enzyme family and on neuronal conduction mechanisms (nicotinic acetylcholine receptor)[18,20–23] at much higher concentrations than the ones we used to inhibit trophozoite growth (Table 1). In a previous report,[18] the anaesthetic EC_{50} value for cyclobutyl methanol (a CBC analogue) on vertebrates was 5.4 mM, 480 times higher than the CBC EIC_{50} value reported here (Table 1), and 6000-fold the concentration we needed to inhibit the ADH activity *in vitro* (Table 2). Mammalian toxicity data on the effects of *n*-alkanols on ion channels indicate that at least 140 mM cyclopropanemethanol and 14 mM cyclobutanemethanol were necessary to inhibit the nicotinic acetylcholine receptor *in vitro*.[22] These values are 3600- and 1250-fold the concentrations used in our study to inhibit 50% of amoebic trophozoite growth by the analogues CPC and CBC, respectively (Table 1). A survey revealed that 1-aminocyclopropanol, an aldehyde dehydrogenase inhibitor, had no inhibitory effect on rat brain tryptophan hydrolase activity *in vitro*.[21] A recent *in vivo* study showed that methyl isobutyl carbinol (MIBC) had low potential for toxicity in male rats, based on the plasma levels found after 12 h of a single 5 mmol/kg oral dose of MIBC.

Neither mortality nor clinical signals of toxicity were observed in the animals.[24] In summary, no study has reported any harmful effect of these or similar compounds on vertebrate dehydrogenases.

In conclusion, our results suggest that CPC and CBC can be used safely and effectively for the treatment of amoebiasis. These compounds are expected to be non-toxic to humans at the concentrations required to eliminate *E. histolytica* trophozoites. As anti-amoebic agents, CPC and CBC appear to specifically target EhADH2, while as anesthetics they act on P450 oxygenases and neuronal receptors. Similarities between EhADH2 and the *Giardia lamblia* AdhE enzyme[17,25] indicate that CPC and CBC could be effective drugs for the treatment of both amoebiasis and giardiasis.

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