“Drug effects and drug targets from human pathogenic amoebas”

Entamoeba histolytica, Acanthamoeba polyphaga and Naegleria fowleri

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INTRODUCTION

In our laboratory we have been working for several years about the searching of thiol compounds in human pathogenic amoebas like *Entamoeba histolytica, Acanthamoeba polyphaga* and *Naegleria fowleri.* Here we present definitive data to show, that the thiol-bimane compound isolated and purified from trophozoites of these three amoebas by HPLC, after characterization by matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF/TOF), corresponds to the characteristic monoprotonated ion of trypanothione-(bimane). Besides we have demonstrated that *E. histolytica* contains the gene for the trypanothione reductase (TR) which was previously supposed to occur mainly in trypanosomatids. Also we analyzed the effects of neuroleptic agents, like chlorpromazine and trifluoperazine; the antimycotics, amphotericin B, ketoconazole and miconazole and four antibiotics, pentamidine, rifampicin, mepacrine and metronidazole on the NADPH-dependent disulfide reducing enzymes cystine reductase (CysR), glutathione reductase (GR) trypanothione reductase (TR) in *E. histolytica, A. polyphaga, N. fowleri.* All nine drugs studied had the capacity to inhibit the putative disulfide reductase from the trophozoites. The presence of the trypanothione/trypanothione reductase system in these amoebas creates the possibility of using this enzyme as a new "drug target" for rationally designed drugs to eliminate the parasite, without affecting the human host.

MATERIALS AND METHODS

Cultures Conditions

E. histolytica trophozoites, strain HK9, was cultured axenically at 37°C for 60 H as described by Diamond et al. The culture medium contained peptone-biotryptase plus 10% (v/v) calf serum and vitamins/Tween 80. *A. polyphaga* was grown at 28–30°C in axenic conditions in liquid medium, 10% inactivated fetal bovine serum (FBS). Highly pathogenic *N. fowleri* ATCC 30808 was grown axenically at 37°C for 60 H in culture medium containing bacto casitone, 2% pancreatic digest of casein, and 10% inactivated fetal bovine serum.

Analysis of low Molecular Mass thiol compounds

The thiol compounds from *E. histolytica, A. polyphaga and N. fowleri* were purified from normal trophozoites (oxidized and reduced forms) using $HClO_4$ and DTT. The reduced forms were extracted and derivatized with acetonitrile/2 mM mBBr followed by HPLC. After neutralization with 4M KOH and elimination of $KClO_4$, the material was passed through a Florisil column. The column had been washed with several volumes of 5% (v/v) acetic acid and then with water until the absorbance of the effluent *A*280 was less than 0.1. The Trypanothione was eluted with 20% (w/v) pyridine. After evaporation, the extract was dissolved in 5 mM ammonium bicarbonate buffer (pH 8.3)

MALDI-TOF/TOF Mass Spectrometry analysis

After RP-HPLC separation of the thiol-bimane compounds, they were desalinated and concentrated using Zip tip C18 column. Samples were applied on a stainless sheet be analyzed using Mass Spectrophotometer MALDI-TOF/TOF 4800 Proteomics Analyzer in reflector positive ion mode, using a laser for ion dissociation. Additionally, some samples corresponding to Trypanothione were analyzed using an electrospray system.

RESULTS

A.- Cold PCA extracts by separate of *E. histolytica*, *A. polyphaga and N. fowleri* cultures were isolated and purified by HPLC and the main compounds identified by MS analysis. The Mass spectrometric analysis of compound Try from the three amoebas obtained by MALDI-TOF MS/MS showed the ion mass of 722.2/ 914.3 and 1104.3 .

B.- The most effective drugs expressed as (IC50) were as follow: the antimycotics ketoconazole and amphotericinB, followed by trifluoperazine, mepacrine, chlorpromazine, miconazole, and metronidazole. The least effectives were rifampicin and pentamidine. The most potent growth inhibitors (MIC100) were the antimycotics amphotericin B and ketoconazole and the neuroleptic trifluoperazine. It was clear that there are major differences between the two amoebas in their susceptibility to some of the drugs.

C.- In relation to the presence of the gene that codifies the enzyme trypanothione reductase (TR) from *E. histolytica* we were able to establish the complete DNA sequence which corresponds up to an 85% to the gene of *Trypanosome cruzi*.

Regarding to the trypanothione reductase activity we were able to show the presence in the three amoebas *E. histolytica, A. polyphaga and N. fowleri.*

CONCLUSIONS

The thiol compounds trypanothione and its precursor glutathione-spermidine, as well as the trypanothione reductase activity which were previously thought to occur only in trypanosomatids, are also present in *E. histolytica*, *A. polyphaga*, *N. fowleri.* The trypanothione/trypanothione reductase system in these amoebas creates the possibility of using this enzyme as a new "drug target" for rationally designed drugs to eliminate the parasite, without affecting the human host.

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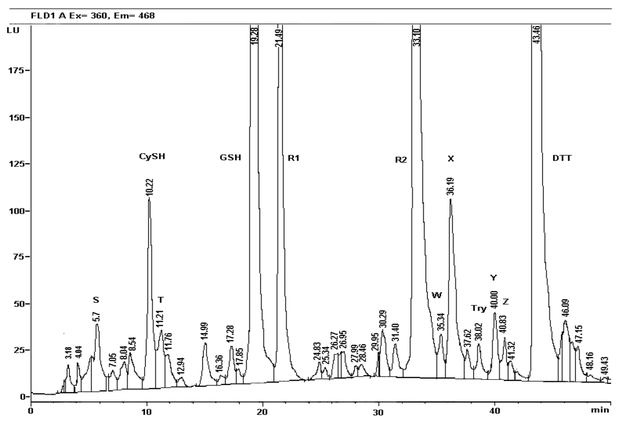
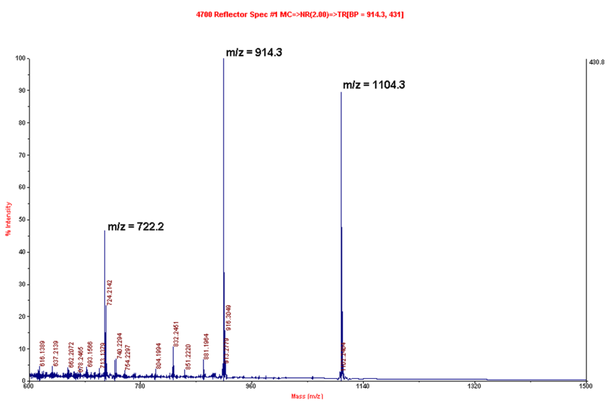
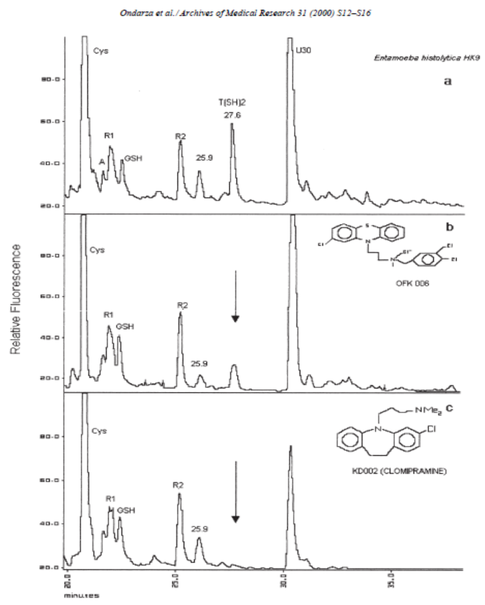
Raúl N. Ondarza, Drug Effects on Drug Targets: Inhibition of Enzymes by Neuroleptics, Antimycotics, Antibiotics and Other Drugs on Human Pathogenic Amoebas and their Antiproliferative Effects, Recent Patents on Anti-Infective Drug Discovery, 2007, Review 2, © 2007 Bentham Science Publishers Ltd.



HPLC elution diagram of thiol compounds from *E. histolytica* HK9. The HPLC separation of thiol compounds was done with a Vydac C18 column using a gradient of acetonitrile/0.1% TFA. Abbreviations: Cys—cysteine; R—reagent; GSH—glutathione; T(SH)2—reduced trypanothione; X, Y, Z—unidentified.

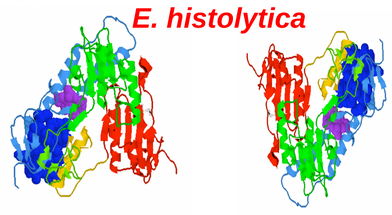


Mass spectrometric analysis of compound Try from *E. histolytica* obtained by MALDI-TOF MS/MS showing the ion mass of 722.2/, 914.3 and 1104.3 .

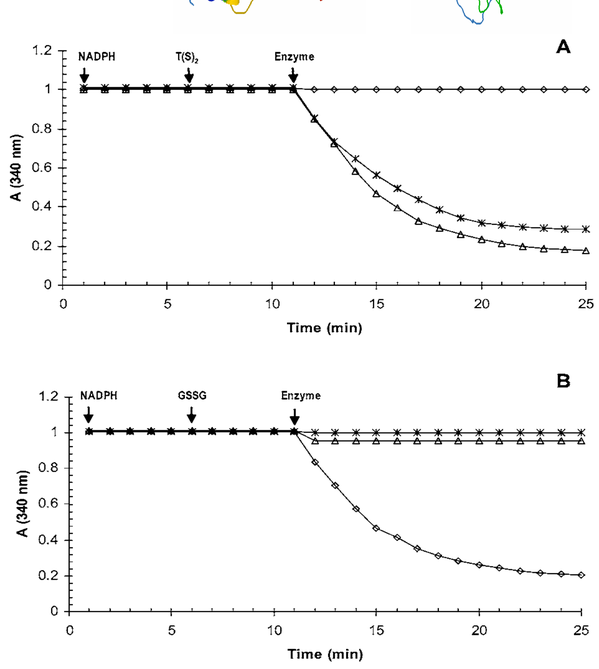


HPLC elution diagram of thiol compounds from *E. histolytica* HK9. a) Normal culture grown for 60 h; b) culture treated for 24 h with 100 mM OFK006, and c) culture treated for 24 h with 100 mM clomipramine.
The HPLC separation of thiol compounds was done with a Vydac C18 column using a gradient of acetonitrile/0.1% TFA. Abbreviations: Cys—cysteine; R—reagent; GSH—glutathione

Trypanothione reductase Dímer of *E. histolytica*



FAD domains are shown colored in dark blue.
The NADPH-binding domains are in green;
The interface domains in red.
And violet to the reducing catalytic site (RCS).



Enzymic activities of TR and GR from partially purified extracts of *E. histolytica* (*), *E. coli* (◊) and *C. luciliae* (Δ) (A) Oxidation of NADPH by *E. histolytica* and *C. luciliae* TR in the presence of $T(S)_2$; *E. coli* had no activity towards oxidized trypanothione. (B) Oxidation of NADPH by *E. coli* GR in the presence of GSSG; *E. histolytica* and *C. luciliae* had no GSSG reductase activity.

Table 2 Effect of five phenothiazine derivatives and five tricyclic neuroleptic drugs on *E. histolytica* HK9 trophozoite proliferation under aerobic conditions

The phenothiazine and tricyclic drugs (100 μM) were added at 36 h of culture for 24 h (up to 60 h of culture). Each value is the mean of duplicates. Barlett's test with $P < 0.05$ versus the control. *Some lysed trophozoites were present.

		Phenothiazine derivative					Tricyclic derivative				
	Control	OFK001	OFK006	OFK008	OFK027	OFK043	KD001	KD002	KD003	KD004	KD005
Cell number ($\times 10^6$)	9.8	5.0*	8.25	2.3*	0.437*	1.125*	4.7	0.81*	2.8	1.8*	4.1

Inhibition of cell proliferation, measured as DNA with Sytox Green of *E. histolytica* HK9 incubated under anaerobic conditions for three time periods with metronidazole (a), clomipramine (KD002:b) diphenidramine (KD005: c) and OFK008 (d) at four different concentrations.