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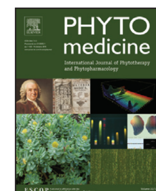

Original article

How do the alkaloids emetine and homoharringtonine kill trypanosomes? An insight into their molecular modes of action

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ABSTRACT

Background: Although *Trypanosoma brucei* causes deadly sleeping sickness, the number of the registered medications is rather limited. Some plant alkaloids are potent trypanocidal agents.
Purpose: In this study, we wanted to elucidate the molecular modes of trypanocidal activity of the alkaloids emetine and homoharringtonine against *Trypanosoma brucei brucei*.
Methods: We investigated the activity of both alkaloids regarding growth recovery from alkaloid-induced stress. We measured the inhibition of protein biosynthesis using the Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay kit. Reduction of mitochondrial membrane potential and cell cycle arrest were measured by means of flow cytometry. Additionally, we determined spectrophotometrically the inhibition of the trypanosome specific enzyme trypanothione reductase activity and DNA intercalation.
Results: Both alkaloids prevented that parasites could resume normal growth after pretreatment with the alkaloids. They inhibited protein biosynthesis in a time- and concentration-dependent manner. In contrast to homoharringtonine, emetine is also a DNA intercalator. Homoharringtonine decreased the mitochondrial membrane potential. Both alkaloids caused cell cycle arrest. Both alkaloids failed to affect trypanothione reductase, a crucial component of the redox system of trypanosomes.
Conclusion: We assume that both alkaloids are primarily inhibitors of protein biosynthesis in trypanosomes, with DNA intercalation as an additional mechanism for emetine. This is the first study that elucidates the molecular mode of trypanocidal action of emetine and homoharringtonine.
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Introduction

Trypanosoma brucei is a unicellular parasitic flagellate, which, if left untreated, causes deadly sleeping sickness in humans and animals. The World Health Organization (WHO) marks the disease as neglected, since it is prevailing in the developing areas of the world, affecting mainly Subsaharan Africa (http://www.who.int/mediacentre/factsheets/fs259/en/ accessed March 2016). The lack of a vaccine puts travelers in this area under risk of infection (Balana-Fouce et al., 2014). The number of medications registered for the treatment of sleeping disease is very limited; only five medications are currently in use: among them suramin and pentamidine that have been in clinical use for more than 70 years (http://www.who.int/mediacentre/factsheets/fs259/en/ accessed March 2016).

Some alkaloids are potent trypanocidal secondary plant metabolites. Two alkaloids with reported high anti-parasitic activity are emetine and homoharringtonine (Krstin et al., 2015; Merschjohann et al., 2001; Rosenkranz and Wink, 2008). Emetine (Fig. 1a) is an isoquinoline alkaloid that has been isolated from *Psychotria ipecacuanha* (Brot.) Stokes (Rubiaceae) and used in folk medicine and phytomedicine as emetic, expectorant and amebicide (Cheong et al., 2011; Wink and Schimmer, 2010). Homoharringtonine (Fig. 1b) is a cephalotaxine derivative that has been isolated from *Cephalotaxus harringtonii* (Knight ex J.Forbes) K. Koch (Taxaceae) and used in traditional Chinese medicine against helminths and malignancies (Al Ustwani et al., 2014; Efferth et al., 2007; Wink and Schimmer, 2010). US Food and Drug Administration (FDA) approved the use of its derivative omacetaxine mepesuccinate for treatment of patients suffering from chronic myeloid leukemia (Chen and Li, 2014). The biological activity of emetine and homoharringtonine is attributed to their ability to inhibit protein biosynthesis, while emetine was also reported to intercalate DNA (Wink and Schimmer, 2010).

Abbreviations: *T. b. brucei*, *Trypanosoma brucei brucei*; FDA, Food and Drug Administration; Rh123, rhodamine 123; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; PBS, phosphate-buffered saline; PI, propidium iodide; TbTR, *Trypanosoma brucei* trypanothione reductase; Tm, melting temperature; EME, emetine; HHT, homoharringtonine; CRK, cyclin related kinase.

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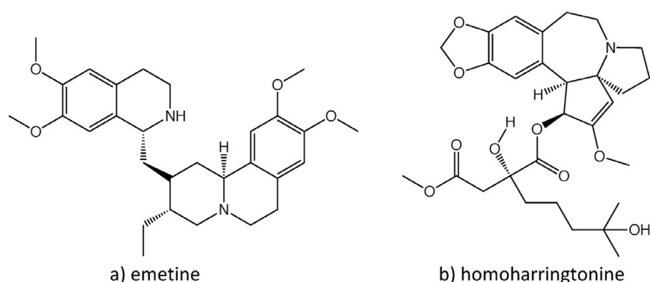

Fig. 1. Chemical structures of emetine (a) and homoharringtonine (b).

Treatment of sleeping sickness lacks innovation; it was 2009, when a combination of nifurtimox and eflornithine was put on the market (http://www.who.int/mediacentre/factsheets/fs259/en/, accessed March 2016). There is therefore a high demand for new drugs to fight sleeping sickness, especially since some strains have developed resistance against common trypanocidal agents (Wink, 2012). Although emetine and homoharringtonine are active against trypanosomes, information of their trypanocidal mode of action is limited. In order to proceed with clinical studies and consequently put these alkaloids in clinical use for the treatment of trypanosomiasis, we require a sound understanding at a molecular level of the way they are killing trypanosomes.

In this study we aimed to elucidate the powerful trypanocidal activity of the alkaloids emetine and homoharringtonine against *Trypanosoma brucei brucei* (*T. b. brucei*). In our previous study (Krstin et al., 2015), where six alkaloids were included, emetine and homoharringtonine showed the highest trypanocidal activity. We investigated the underlying mode of action regarding their influence on protein biosynthesis, mitochondrial membrane potential, cell cycle, activity of the trypanosome-specific enzyme *Trypanosoma brucei* trypanothione reductase and DNA intercalation. This is the first study that clarifies the molecular mode of action of emetine and homoharringtonine in trypanosomes, which should help to infer potential side effects.

Material and methods

Chemicals

Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay kit, MEM medium, methionine-free DMEM medium, non-essential amino acids (NEAA), penicillin, streptomycin and L-glutamine were purchased from Invitrogen, Germany. Cycloheximide (≥ 93%), fetal bovine serum (FBS), dimethylsulfoxide (DMSO; ≥ 99.9%), HEPES (≥ 95%), glucose (≥ 95%), sodium pyruvate, hypoxanthine (98%), thymidine (99–100%), adenosine, bathocuproinedisulfonic acid disodium salt, ethidium bromide, emetine dihydrochloride hydrate, homoharringtonine (≥ 98%), carbonyl cyanide 3-chlorophenylhydrazone (> 97%), NADPH, propidium iodide (≥ 94%), rhodamine 123 and β-mercaptoethanol were obtained from Sigma-Aldrich GmbH, Steinheim, Germany. RNAse A was acquired from Applichem GmbH, Darmstadt, Germany and Lambda DNA from Thermo Fisher Scientific, Karlsruhe, Germany.

Cell culture

We used a *T. b. brucei* TC 221 derived from stock 427 bloodstream cell line- pathogenic only for animals, which justifies its use in laboratories; the strain was acquired from Prof. Peter Overath (MaxPlank Institut für Biologie, Tübingen, Germany). It was maintained in complete Baltz medium (Baltz et al., 1985) and cultivated at 37 °C, 5% CO_2 and 95% humidity. All experiments were performed with cells being in their logarithmic growth phase.

Recovery assay

The assay was executed as previously described with minor modifications (Kessler et al., 2013). A cell density of 1.3×10^6 cells/ml was exposed to different doses of homoharringtonine (0.01, 0.25 and 1 µM) and emetine (0.02, 0.25 and 2 µM) for short periods of time (30, 120 and 240 min). Cells were then centrifuged at $1800 \times g$ for 5 min at room temperature, washed two times with sterile phosphate-buffered saline (PBS) and transferred to fresh, drug-free Baltz medium at a density of 10^4 cells/ml. The cell density was determined every 24 h in the improved Neubauer counting chamber. Growth recovery was monitored for 72 h after drug contact.

Protein biosynthesis inhibition

To investigate the inhibition of global protein biosynthesis in trypanosomes, Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay kit was used. The assay was performed following the kitś instructions. Briefly, 2×10^6 *T. b. brucei* cells/ml were incubated with emetine (0.02, 0.06 and 0.12 µM) and homoharringtonine (0.01, 0.25 and 1 µM) for 4, 8 and 24 h. From this section onwards, lower concentrations of emetine were used than in the recovery assay because of emetine's high toxicity and longer incubation time. After washing, the cells were incubated for 30 min in a methionine-free medium with 50 µM of L-azidohomoalanine-an amino acid analog of L-methionine containing an azido moiety, which is being incorporated into proteins during protein biosynthesis. Afterwards, the cells were washed, fixed and permeabilized. The cells were then incubated with Click-iT® reaction cocktail for 30 min, at room temperature, protected from light. The cocktail contained AlexaFluor 488 conjugated alkyne that detected (by "click" reaction) the amino analog in the azido modified protein. The resulting fluorescence was measured at excitation/emission wavelengths of 485/535 nm using a Tecan Safire Infinite F200 microplate reader (Tecan Crailsheim, Germany). The antibiotic cycloheximide, a known inhibitor of protein synthesis, was used as a positive control.

Mitochondrial membrane potential assay

Decrease in the potential of the mitochondrial membrane ($\Delta\Psi$m) was measured using the fluorescent probe Rh123, which accumulates within mitochondria (Divo et al., 1993). Briefly, 2×10^6 *T. b. brucei* cells/ml were incubated with emetine (0.02, 0.06 and 0.12 µM) and homoharringtonine (0.01, 0.25 and 1 µM) for 4 and 24 h. After washing, cells were incubated with 10 µg/ml Rh123 (Rhodamine 123) on 37 °C for 15 min to verify changes in $\Delta\Psi$m. Data acquisition and analysis were performed at excitation/emission wavelengths of 488/530 nm using FACSCalibur™ flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) equipped with CellQuest™ software. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as a positive control. A total of 10,000 events were acquired in the region that was previously established as the one that corresponded to the parasites.

Cell cycle arrest

To determine whether the alkaloids arrest the cell cycle of trypanosomes, emetine (0.02, 0.06 and 0.12 µM) and homoharringtonine (0.01, 0.25 and 1 µM) were incubated with 10^6 *T. b. brucei* cells/ml for 4 and 24 h at 37 °C. Afterwards, the cells were washed two times with PBS and fixed overnight with 70% EtOH

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