Experimental Parasitology xxx (2018) 1–10

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Ultraestructural study of effects of alkylphospholipid analogs against nematodes

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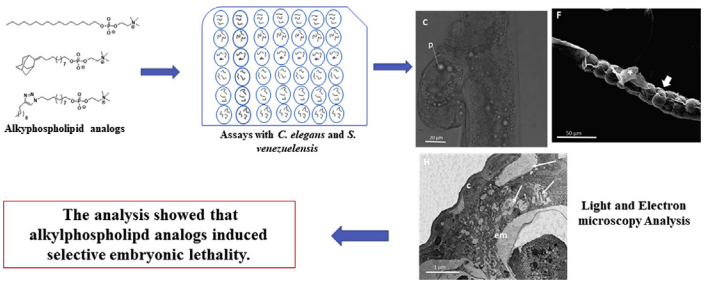
HIGHLIGHTS

- The alkylphospholipids, miltefosine, TCAN26, and TC70 affect *C. elegans* adults.
- Miltefosine affects *C. elegans* and *S. venezuelensis* larvae.
- Miltefosine induces several alterations in the body wall, reproductive system, and embryos of *C. elegans*.
- The ultrastructural effects of miltefosine are similar in both *C. elegans* and *S. venezuelensis*.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 December 2017
Received in revised form
5 February 2018
Accepted 24 February 2018
Available online xxx

Keywords:
Miltefosine
Anthelmintic
Morphology
Ultrastructure

ABSTRACT

Alkylphospholipid analogs were initially developed as anticancer agents and were later found to antiparasitic activity. Miltefosine is the prototype alkylphosphocholine and is the first oral treatment against visceral leishmaniasis. Here we investigated the effects of miltefosine and two ring-substituted alkylphosphocholine derivatives, TCAN26 and TC70, on the viability, morphology, and ultrastructure of the life stages of *Caenorhabditis elegans* and infective larvae of the parasite *Strongyloides venezuelensis*. Miltefosine displayed activity against *C. elegans* adults at low concentrations and was more effective than TCAN26 and TC70. Miltefosine inhibited the hatching of eggs, leading to embryonic lethality, and showed larvicidal activity against *C. elegans* and *S. venezuelensis* larvae after 24 h. Mitelfosine also induced alterations in the reproductive system of hermaphrodites, causing vulvar prolapse and general effects in the body wall. Electron microscopy analysis showed that miltefosine induced selective embryonic lethality, leading to cell death. Our results suggest that alkylphospholipid analogs are a potential new alternative for anti-nematode chemotherapy.

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https://doi.org/10.1016/j.exppara.2018.02.004
0014-4894/

1. Introduction

Intestinal parasitic infections are a serious problem in underdeveloped countries and are usually associated with, and aggravated by, poor sanitation and lack of information, causing high morbidity rates and a range of health problems (Frei et al., 2008). In ruminants, gastrointestinal nematodes cause disease, death, and decrease the production of milk, meat, and wool, resulting in economic losses and ecological impact (Bekele et al., 1992; Zainalabidin et al., 2014).

The benzimidazoles and the nicotinic acetylcholine receptor agonists are the two main classes of drugs used to control intestinal nematodes. However, resistance to these compounds is a serious problem in the control of helminthiasis (Keiser and Utzinger, 2008), thus necessitating the development of new drugs. In this context, *Caenorhabditis elegans*, a free-living nematode, has been identified as an excellent model for the screening of compounds with potential anthelmintic activity (Simpkin and Coles, 1981).

Alkylphospholipid analogs (APs) are known as antitumor lipids. APs have amphiphilic structures that resemble those of natural phospholipids and are incorporated preferentially in the membranes of tumor cells (Gajate and Mollinedo, 2002). The most widely studied AP is miltefosine, which is used as the first oral treatment for visceral leishmaniasis (Chrusciak-Talhari et al., 2011; Machado et al., 2010). In recent years, studies have shown that APs are active against several pathogenic protozoa.

Miltefosine was studied against *Trypanossoma cruzi* and *Trypanosoma brucei* species. The *in vitro* and *in vivo* studies have demonstrated that this drug is more active against *T. cruzi* than the standard drugs, nifurtimox and benznidazole (Croft et al., 1996). The miltefosine induced the antiproliferative effect on *Trichomonas vaginalis* causing several ultrastructural alterations indicative of cell death by apoptosis (Blaha et al., 2006; Rocha et al., 2014) and the miltefosine is also effective against *Giardia lamblia*, causing severe morphological alterations in trophozoites, mainly at the level of cell membrane and adhesive disc. The results obtained by *in vitro* and *in vivo* studies demonstrated that miltefosine is found to be superior to metronidazole, the standard drug used for treatment of giardiasis (Eissa and Amer, 2012).

The APs had potential fungicidal activity. Studies showed that the miltefosine was effective against biofilms of *Fusarium oxysporum* and *Candida albicans* (Machado Vila et al., 2013). *Sporothrix brasiliensis* exposure to miltefosine presented in ultraestructural analysis loss of plasma membrane integrity, decrease in cytoplasmic electron density, alterations in the thickness of cell wall layers and accumulation of an electron-dense material in the cell wall (Borba-Santos et al., 2015).

These molecules are promising candidates in the search for anthelmintics. Several studies showed that the miltefosine is active against *Schistossoma mansoni* (Eissa et al., 2011; Bertão et al., 2012; El-Moslemany et al., 2016). The miltefosine is effective against adults, aquatic stages of *S. mansoni* and its snail *Biomphalaria alexandrina*, which favors the control of the disease. The same is seen with different stages of *Schistossoma haematobium* and its intermediate host, the *Bulinus truncutes* (Eissa et al., 2011). In ultrastructural observation, the miltefosine induced in *S. mansoni* adults the membrane destruction, disintegration of the tubercles, spine reduction and erosion, blister formation and rupture, and the emergence of holes (Bertão et al., 2012).

The nematode model *Caenorhabditis elegans* when exposed to APs as edelfosine, perifosine, erucylphosphocholine and miltefosine, presented a selective and direct killing action on *C. elegans* embryos, especially with edelfosine (Sánchez-Blanco et al., 2014). Recently it was also reported the *in vitro* and *in vivo* activity of edelfosine, miltefosine, perifosine against *Strongyloides venezuelensis*. It was observed that the edelfosine displayed the highest activity probably causing through induction of apoptosis-like cell death (Legarda-Ceballos et al., 2016).

Based on previous studies, we decided to analyze the activity of three alkylphospholipid analogs against *C. elegans* adults using a microscopy approach. The nematode activity of the most active compound was analyzed in embryonated eggs, larvae of *C. elegans*, and infectivity larvae of *Strongyloides venezuelensis*, a parasitic nematode of rats that is frequently used as a model to study human and animal strongyloidiasis.

2. Material and methods

2.1. Maintenance of Caenorhabditis elegans and Strongyloides venezuelensis

C. elegans (Wild-type N2), was obtained from the *Caenorhabditis* Genetics Center, was grown at 20 °C on NGM (nematode growth medium: 2.5 g casein peptone, 3 g NaCl, 17 g agar, 0.5% cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 25 mM KH_2PO_4/K_2HPO_4 per liter of water) seeded with *Escherichia coli* strain OP50 as described by Brenner (1974).

Male *Rattus norvegicus* (Wistar) were subcutaneously infected with 1.500 third-stage infective larvae (L3) of *S. venezuelensis*. After eight days, fecal samples were collected and placed on charcoal culture at 38 °C. The infective larvae went removed from the cultures after three days by Rugai's method. The rats were handled in compliance with the ethical guidelines adopted by the Comissão de Ética no Uso de Animais (CEUA) of the Universidade Federal do Rio de Janeiro (UFRJ). The experiments were conducted in accordance with animal ethics guidelines and were approved by the local Ethical Committee (protocol IBCCF20409/16).

2.2. Drugs

Miltefosine (Cayman Chemical Company, MI, USA) and two synthetic analogs (Fig. 1) were evaluated for anthelmintic activity. Compounds TCAN26 and TC70 were synthesized as previously described by Avlonitis et al. (2003) and Calogeropoulou et al. (2008).

Miltefosine was diluted in distilled water and the analogs TCAN26 and TC70 were diluted in DMSO: ethanol (1:1) from Merck. DMSO in the medium never exceeded 0.1% (v/v) and had no effect on the survival of nematodes. The stock solutions were maintained at −20 °C.

2.3. Anthelmintic assays

2.3.1. Assays with C. elegans adults

The *C. elegans* population was synchronized by incubating the culture with lysing solution (5 M NaOH and 1% hypochlorite), and collecting just the eggs. After three days, the nematodes originated had the same age. They were collected by washing with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, and 0.25 g $MgSO_4 \cdot 7H_2O$ per liter of water) and centrifuged at 800 × g for 5 min. The supernatant was removed and the helminths were washed in the same buffer.

Approximately thirty adults nematodes were incubated in S medium [(S basal 5.85 g NaCl, 1 g K_2 HPO_4, 6 g KH_2PO_4, 1 ml cholesterol (5 mg/ml in ethanol), H_2O to 1 L) 10 ml potassium citrate 1 M pH 6, 10 ml trace metals solution, 3 ml $CaCl_2$ 1 M, 3 ml $MgSO_4$ 1 M per liter of water] supplemented with *E. coli*. Different concentrations (1–200 μM) of miltefosine, TCAN26 e TC70 were added just once in the beginning of treatment. A group without treatment was used as a negative control and a group with 0.01% DMSO (solvent control) was formed in all experiments.

Please cite this article in press as: Sant'Anna, V., et al., Ultraestructural study of effects of alkylphospholipid analogs against nematodes, Experimental Parasitology (2018), https://doi.org/10.1016/j.exppara.2018.02.004