The alkylphospholipid edelfosine shows activity against *Strongyloides venezuelensis* and induces apoptosis-like cell death

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Abstract

Strongyloidiasis is widely distributed in the tropical and subtropical areas. Ivermectin is the drug of choice for the treatment. However, The concerns about relying treatment on a single drug make identification of new molecules a priority. Alkylphospholipid analogues, including edelfosine, are a group of synthetic compounds that have shown activity against protozoan parasites and also against the helminth parasite *Schistosoma mansoni*. The activity of edelfosine, miltefosine, perifosine against *Strongyloides venezuelensis* was assessed both in cultures of third-stage larvae (L3) and infected mice. The induction of an apoptosis-like mechanism in larvae after treatment was studied. Larval motility and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) assay were used to evaluate antiparasitic drug efficacy in L3 cultures as screening test. Edelfosine displayed the highest activity against L3 and the best selectivity index (LD_{50} = 49.6 ± 5.4 µM, SI = 1.1) compared to miltefosine or perifosine activity. L3 after culture with edelfosine were not able to develop an infection in CD1 mice. The oral treatment with edelfosine showed reduction of 47% in parasitic females allocated in the gut. Moreover, DNA fragmentation was also observed by TUNEL staining in edelfosine treated L3. These data suggest that edelfosine could be an effective drug against strongyloidiasis, probably through apoptosis-like cell death.

1. Introduction

Strongyloidiasis is caused by nematodes of the genus *Strongyloide*s widely distributed in tropical and subtropical areas. There are about 52 species in the genus, but only *S. stercoralis* and *S. fuelleborni* infect humans. It is estimated that 30 to 100 million people are infected worldwide (Bisoffi et al. 2013, Puthiyakunnon et al. 2014). Infective third-stage larvae (L3) penetrate the skin, migrate to the lungs, reaching the trachea, oesophagus and small intestine where became mature. Parthenogenetic females lay eggs that hatch into rhabditiform larvae (L1), which are eliminated in the faeces. Some L1 remain and molt into L3 establishing reinfection cycles. The clinical strongyloidiasis ranges from asymptomatic infection, cutaneous *larva migrans*, Löeffler syndrome, chronic intestinal infection to life-threatening disseminated hyperinfection depending upon the immune status of the patient and the presence of risk factors, such as corticosteroid therapy, stem-cell transplantation, alcoholism or HTLV-1 infection. Disseminated hyperinfection involves a massive spread of the parasite in situations of immunosuppression with high mortality (Montes et al. 2010, Schar et al. 2013, Sharifdini et al. 2014).

Thiabendazole was long the drug of choice for treating strongyloidiasis. However, it is no longer available due to the strong adverse side effects. Albendazole, another broad- spectrum drug and anthelminthic agent is effective against *S. stercoralis*. Currently, ivermectin is the best therapeutic option for the treatment of strongyloidiasis, with cure rates from 70% to 85% of chronically infected patients (Pitisuttithum et al. 1995, Igual-Adell et al. 2004, Stuart et al. 2009). The anti-*Strongyloides* activity of ivermectin is superior to that of albendazole (Suputtamongkol et al. 2011).

Alkylphospholipid (APL) analogues include edelfosine, miltefosine and perifosine, a heterogeneous group of natural lipids (Fig. 1) with promising anticancer activity. These

compounds act at the level of cell membranes affecting apoptotic signalling. Edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) is considered the prototype APL molecule and a promising antitumor ether phospholipid drug that acts by activating apoptosis through its interaction with cell membranes (Gajate and Mollinedo 2002, Mollinedo et al. 2004, Gajate and Mollinedo 2007). In addition to its antitumor activity, edelfosine exerts cytotoxic activity against parasitic protozoa such as *Leishmania* spp (Varela et al. 2012), *Trypanosoma cruzi* (Luna et al. 2009), *Trichomonas vaginalis* (Rocha et al. 2014), *Giardia lamblia* (Eissa and Amer 2012), *Acanthamoeba keratitis* (Polat et al. 2012), *Neospora caninum* (Debache and Hemphill 2012), *Babesia* spp and *Theileria equi* (AbouLaila et al. 2014). These compounds are also active against larval stages and eggs of the free-living nematode *Caenorhabditis elegans* (Sanchez-Blanco et al. 2014) and the blood fluke *Schistosoma mansoni* (Eissa et al. 2011, Bertao et al. 2012, Yepes et al. 2014). The human parasite *S. stercoralis* cannot complete its development in immunocompetent mice and rats. Thus, attention has been focused on the related parasites *Strongyloides ratti* and *Strongyloides venezuelensis*, parasite of rats, to study host-parasite relationships (Sato and Toma 1990), and as general experimental models of intestinal parasitism (Yasuda et al. 2014).

In the present study, the efficacy of alkylphospholipid derivatives in killing *S. venezuelensis* was investigated using *in vitro* and *in vivo* approaches, comparing it to other APLs and assessing its potential as a chemotherapeutic alternative for the treatment of strongyloidiasis. Insights into the mode of action of edelfosine in killing *S. venezuelensis* larvae were also investigated using an *in vitro* approach.

2 Materials and Methods

2.1. Ethics statement

The animal procedures in this study complied with the Spanish (L 6/3013, RD 53/2013) and European Union (Di 2010/63/CE) regulations regarding animal experimentation for the protection and humane use of laboratory animals. The University of Salamanca's accredited Animal Experimentation Facilities (Registration number PAE/SA/001) were used for these procedures. The University of Salamanca's Ethics Committee also approved the procedures that were used in this study (Permit Number: 8402). The animals' health and welfare status was monitored throughout the experiments by a health surveillance program according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines and also by the University of Salamanca's standardized protocols. All efforts were made to minimize suffering.

2.2. Animals

Sixty-nine six-week-old CD1 mice weighing 25-30 g and twelve male Wistar rats weighing 150-200 g (Charles River Laboratories, Barcelona, Spain) were used for *in vivo* experiments and life cycle maintenance, respectively. Animals were maintained in the University of Salamanca's Animal Care Facilities and kept in standard polycarbonate and wire cages with food and water *ad libitum* with regular 12 h light–dark periods and 20-22°C temperature. Animals at the end of the experimentation or those presenting any deterioration of the health status were humanely euthanized by intraperitoneal injection of a lethal dose of pentobarbital (100 mg/kg). Size of groups was calculated by power analysis (Charan and Kantharia 2013) using the "size.fdr" package for R and following the 3Rs recommendations (Festing and Altman 2002).

2.3. Drugs

Edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocoline) was obtained from R. Berchtold (Biochemisches Labor, Bern, Switzerland). Miltefosine (hexadecylphosphocholine) was from Calbiochem (Cambridge, MA). Perifosine (octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate) was from Zentaris (Frankfurt, Germany). Stock sterile solutions of the distinct APLs (2 mM) were prepared in RPMI-1640 culture medium (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin, as previously described (Mollinedo et al. 1997). Ivermectin was purchased from Sigma Aldrich and diluted in dimethyl sulfoxide (DMSO) at a final concentration of 10 µM.

2.4. *S. venezuelensis* life cycle maintenance and parasitological techniques

The *S. venezuelensis* strain from Department of Parasitology, Federal University of Minas Gérais (Brazil) was maintained by serial passage in Wistar rats at the University of Salamanca's animal care facilities since 2003, according to the procedure described by Martins et al. (Martins et al., 2000). Briefly, rats were subcutaneously infected with 6000 third stage larvae (L3) in 500 µL of phosphate buffered saline (PBS) using a 23-gauge needle syringe. Faeces from infected rats (5-14 days p.i) were cultured with vermiculite and water at 28ºC for 3-4 days and then L3 were recovered using a Baermann apparatus. Larvae were decontaminated by mild sodium hypochlorite treatment and exposure to an antibiotic and antifungal cocktail according to Martins et al. (2000). Absence of bacteria was confirmed by culturing L3 on a Petri plate containing blood- agar at 28 ºC during 24 h. Faecal egg counts were performed by

placing animals individually on grids over clean, moist absorbent paper and allowing them to defecate. Individual faecal samples were collected, preserved in a 10% formalin buffered solution and eggs were counted in triplicate samples using the McMaster technique. The upper halves of the small intestines of experimental rats were removed at necropsy, cut longitudinally, minced and placed in a sedimentation cup wrapped by 8 layers of gauze in phosphate buffered saline for two hours at 37 °C. Parasitic females were collected from the sediment and counted.

2.5. Cytotoxicity in murine macrophages

For cytotoxicity assays the mouse-derived peritoneal macrophage cell line J774.2 (Sigma-Aldrich) was used. Cells were maintained at 37 °C with a 5% CO_2 atmosphere and cultured in plastic culture flasks with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (Sigma-Aldrich), 2 mM glutamine (Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich) and 100 µg/mL streptomycin (Sigma-Aldrich). 200 µL of a suspension containing 2 x 10^5 macrophages/mL were added onto 96 well flat-bottom microplates (NUNC) and allowed to incubate for 2 h. After, APL's were added at different concentrations ranging from 1 to 100 µM for the next 48 h and 50 µL of XTT were added to each well and incubated again for 24 h at 37 ºC, 5% CO_2. Finally, 100 µL of DMSO (Sigma-Aldrich) were added to each well and the absorbance was measured at 492 nm using an ELISA-plate reader (Anthos 2010; Anthos Labtec Instruments, Wals, Austria). Ivermectin-treated, untreated and DMSO-treated macrophages were used as controls. Each concentration was assayed in triplicate in three independent experiments and the IC_{50} value for each APL was calculated by sigmoidal regression analysis (Gomez-Ayala et al. 2010).

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167 **2.6. *In vitro* activity of alkylphospholipids**

168 A batch of 7000 *S. venezuelensis* L3 were rinsed twice with PBS and 100
169 larvae per well were distributed in 24-well flat bottom culture plates. Larvae were
170 incubated at 28 ºC for 2 h to allow adaptation, and treated with edelfosine, miltefosine
171 or perifosine in the range of 1 to 100 µM for 72 h as screening test (Keiser et al.
172 2008, Tritten et al. 2011, Olounlade et al. 2012). Mortality was assessed as the lack of
173 any movement detected during 2 min of observation under the microscope
174 (magnification 4x), at 24, 48 and 72 h after treatment. Video recordings were taken
175 using an AM423 camera and DinoCapture software version 2.0, (Dino-Lite Digital
176 microscope, Naarden, Holland). Larvae were considered dead when no movement was
177 detected for at least two minutes of detailed examination. As controls, *S. venezuelensis*
178 larvae were incubated in the presence of PBS or treated with ivermectin 10 µM. All
179 experiments were carried out in triplicate and performed at three different times. The
180 colorimetric XTT assay was also used for measuring larvae viability (Paull et al. 1988).
181 After treatment, *S. venezuelensis* L3 were incubated with 50 µL of XTT for 24 h and
182 the absorbance was measured at 492 nm using an ELISA-plates reader (Anthos 2010;
183 Anthos Labtec Instruments, Wals, Austria). Untreated, heat-killed and ivermectin-
184 treated *S. venezuelensis* L3 were used as controls. The antiparasitic activity of the
185 compounds was expressed as the concentration able to kill 50 % of larvae (LD_{50}) and it
186 was calculated by sigmoidal regression analysis (Gomez-Ayala et al. 2010). The
187 Selectivity Index (SI = mammalian cell IC_{50}/ Larva LD_{50}) for each compound was
188 calculated to compare the strongyloidicidal activity with its respective mammalian cell
189 cytotoxicity.

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2.7. Viability of edelfosine-treated-L3 in mice

S. venezuelensis L3 were rinsed twice with PBS and 100 larvae were distributed per well in 24-well flat bottom culture plates. Larvae were incubated with 5, 10, 20 and 40 µM of edelfosine for 24 h at 28 ºC. Untreated-L3 and ivermectin-treated-L3 were used as controls. After incubation period larvae were carefully recovered, re-suspended in PBS and used to subcutaneously infect thirty-six CD1 mice randomly distributed in six groups with six animals each. Parasite eggs in faeces were monitored on days 5, 6 and 7 p.i to assess the ability of edelfosine-treated-L3 to infect mice.

2.8. Edelfosine activity against S. venezuelensis infection in mice

Two different experiments were performed to assess the *in vivo* activity of edelfosine. In the first experiment, mice were randomly distributed in three experimental groups with 5 mice per group as follows: Infected control; Infected and treated with ivermectin; Infected and treated with edelfosine. Ivermectin was administered orally at 0.2 mg/kg/day once on day 5 p.i. and edelfosine was administered at 20 mg/kg/day from the day of the infection to day 5 p.i. since migrant larvae and adults are present in human strongyloidiasis. All animals were infected by subcutaneous injection with 3000 L3 of *S. venezuelensis* resuspended in PBS. Edelfosine was administered to reach the therapeutical concentration synchronically with ivermectin after day 5 p.i following previous studies (Yepes et al. 2014). Parasitic females were recovered from the gut and eggs in faeces were counted on day 7 p.i using groups of five animals. In the second experiment, mice were randomly distributed in three experimental groups with six mice per group with the same groups as above and we conducted faecal egg counts on days 5, 7, 9, 11, 15, 17 and 19 p.i.

2.9. Assessment of apoptosis-like cell death by TUNEL assay

DNA fragmentation, which usually occurring in apoptosis-like cell death was analysed by the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) technique as previously described (Gajate et al. 2009). Larvae were washed with PBS, centrifuged at 1500 rpm for 3 min, mounted on poly-L-lysine-coated microscope slides (Menzel-Gläser, Braunschweig, Germany) and incubated for 24 h at 37 ºC to stick the L3 to the slides. Larvae were then fixed in 4% paraformaldehyde (Sigma, St. Louis, MO) for 25 min, and washed with PBS as above. Fixed parasites were treated with trypsin and 0.25% EDTA, and stained for fragmented DNA using the Fluorescein Apoptosis Detection System (Promega, Madison, WI) according to the manufacturer's instructions. Propidium iodide was added for 15 min to stain nuclei of both apoptotic and non-apoptotic cells in red, whereas fluorescein-12-dUTP was incorporated at the 3′- OH ends of fragmented DNA resulting in localized green fluorescence within the nuclei of apoptotic cells. Samples were analysed with a Zeiss LSM 510 laser scan confocal microscope.

2.10. Statistical analysis

Data are expressed as mean and standard deviation (SD). Test for normality was performed by Kolmogorov-Smirnov and homogeneity of variance was tested by the Barrett test. Then one-way ANOVA analysis of variance, followed by Tukey's honest significance (HDS) test was performed to determine any statistical differences between treated and untreated controls. Differences were considered statistically significant at $p < 0.05$. The data were processed using GraphPad Prism 5 (GraphPad Software, San Diego, CA) for Mac.

3. Results

3.1. Experimental infection and treatments

All mice used in this study (69/69) remained alive during the whole time-course experiments. According to our health surveillance program the status health of all animals used here was optimal during the experimentation. Any symptoms of severe pain, excessive distress, suffering or an impending death were observed in any of the animals. The success of the experimental infection was verified in each mouse by the observation of larvae released in the faeces, demonstrating that all animals were infected. Daily oral administration of 20 mg/kg edelfosine and 10 mg/kg ivermectin were well tolerated by CD1 mice in all experiments.

3.2. Cytotoxicity in murine macrophages

Cytotoxicity of each compound was evaluated the in macrophage cell line by calculating the IC_{50} for each APL, at six different concentrations. APL cytotoxicity was ranked as follow: edelfosine (IC_{50} 53.4 ± 5.3 µM) < perifosine (IC_{50} 52.8±6.3 µM) < miltefosine (IC_{50} 27.3 ± 3.8 µM), thus showing that edelfosine is the less toxic compound to the macrophages (Table 1).

3.3. In vitro activity of APLs against S. venezuelensis third stage larvae

First, *in vitro* the anti-*Strongyloides* activity of edelfosine, miltefosine and perifosine were analysed through XTT assay after 72 h of incubation using L3 cultures with a range of 1-100 µM. Edelfosine showed the highest efficacy in killing the parasite, being effective at ≥ 40 µM with a LD_{50} value of 49.6±5.3 µM evaluated by the XTT assay (Fig. 2). It would be necessary to administer edelfosine 80 or 100 µM to attain efficacy similar activity to that of ivermectin, the drug of choice for

treating strongyloidiasis (LC50 = 0.41 ± 0.12 µM). Miltefosine and perifosine also showed efficacy in killing the parasite at higher concentrations, but their anti-*Strongyloides* activity seems rather poor as compared to either edelfosine or ivermectin (LD_{50} = 85.7 ± 8.7 µM and LD_{50} = 90.6 ± 10.5 µM, respectively; Fig. 2). Edelfosine also showed the highest Selectivity Index as demonstrated by the SI value, ranked as follows: edelfosine (SI 1.1) > perifosine (SI 0.6) > miltefosine (SI, 0.6).

3.4. Dose- and time-dependent effects of APLs on S. venezuelensis larvae

How the dose and the time of action of APL derivatives affected the parasites were investigated and microscopic observation of larvae incubated was performed at 24, 48 and 72 h to determine decrease in motor activity. As shown in S1-S3 videos, edelfosine's activity is time and dose-dependent, reaching its maximum efficacy at 72 h showing mortality of *S. venezuelensis* L3 equal to 92% at 80 µM and 100% at 100 µM. These results were similar to those obtained with ivermectin at 10 µM. At 48 h significant efficacy of edelfosine (about 50%) was also found with 80 and 100 µM (videos S1-S3). Miltefosine's activity was also time and dose dependent and it was inferior to that of edelfosine, showing its maximum activity at 100 µM being close to 54% of reduction in larvae motility (videos S4-S6). Perifosine was the ALP with the lowest activity against larvae. Only at higher concentrations (≥ 80 µM) and after 72h of treatment perifosine reached a significant reduction of larvae motility (66%) (videos S7-S9). Untreated *S. venezuelensis* larvae remained alive during the next 72h after being cultured, reaching a motility of around 92%, whilst ivermectin-treated larvae were completely killed at 10 µM after 24h of treatment.

3.5. Edelfosine treated-L3 are not able to infect mice.

In order to complete the assessment of the lethal effect of edelfosine L3 cultures were treated with increasing concentrations of the drug, and after 24 hours they were used to infect mice. Egg laying was monitored until day 7 p.i. It was observed that L3 treated with edelfosine at 20-40 µM for 24 hours were unable to develop patency in mice because no-eggs were found in faeces of mice challenged with these larvae. Similarly, mice challenged with ivermectin-treated-L3 (Fig. 3). In contrast, animals treated with 1-10 µM developed patent infections but with a significant reduction in eggs per gram of faeces compared to infected controls (Fig. 3).

3.6. Edelfosine *in vivo* activity in mice infected by *S. venezuelensis*

CD1 mice were subcutaneously infected with 3000 *S. venezuelensis* larvae and then orally treated with edelfosine during five consecutive days in order to reach a therapeutic level of the drug after day 5 p.i. when larvae are finishing their migration and reaching maturity and first eggs appear in faeces. In the first experiment, a significant reduction of 47% was observed in parthenogenetic females recovered from small intestines of edelfosine-treated mice on day 7 p.i (350 ± 162 females) compared to infected controls (661 ± 259 females). A non-significant reduction in faecal egg counts (23 %) was observed on day 7 p.i (65577 ± 10402 EPG *cf* 85171 ± 41012 EPG; Fig. 4). In the second experiment to assess the efficacy on egg laying curves significant reductions of faecal egg counts were observed from day 9 to the end of the experiment (Fig. 5).

3.7. Assessment of apoptosis-like cell death by TUNEL assay

The above results suggested that edelfosine induced killing of *S. venezuelensis* larvae. *S. venezuelensis* L3 larvae treated with 100 µM edelfosine for 72h were analysed for the induction of a putative apoptosis-like death by using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) technique for detecting DNA fragmentation. Labelling of the 3′-OH ends of fragmented DNA with fluorescein-12-dUTP allowed visualization of apoptotic-like cells. Cells were permeabilized and stained with propidium iodide to visualize nuclei from both non-apoptotic and apoptotic cells in red, whereas TUNEL-positive cells, representing apoptotic cells, were stained in green. As shown in Fig. 6, a potent and extensive DNA fragmentation along the whole parasite was found, suggesting an apoptosis-like response in edelfosine-treated *S. venezuelensis* L3 larvae along the whole parasite.

4. Discussion

The present study provides the first insights into the chemotherapeutic potential of the edelfosine against the infection caused by the nematode *S. venezuelensis* in a murine model. The study employed a series of *in vitro* and *in vivo* experiments and also reveals an approximation to its mode of action using the TUNEL assay. The APLs are a group of molecules, which have shown a wide variety of biological functions and have also been assayed against several protozoa, nematodes and trematodes. There are not previous reports in the literature concerning the use APLs for the treatment of strongyloidiasis. Here edelfosine showed the more potent activity against larval *S. venezuelensis in vitro* than other APLs ranked as follows: edelfosine > miltefosine > perifosine. It was also demonstrated that the efficacy of edelfosine in *S. venezuelensis* infections in mice. Although its activity is not as effective as ivermectin, currently the

primary drug for treatment of strongyloidiasis, our data suggests that oral treatment with edelfosine decreases significantly both the number of eggs per gram of faeces and the number of parasitic female worms in the gut of mice. This efficacy is in concordance with the schistomicidal activity against adults *in vitro* and *in vivo* and the reduction of tissue egg burden in mice treated orally with edelfosine (Yepes et al. 2014). Moreover, a combination of edelfosine and praziquantel has demonstrated efficacy against schistosomula (Yepes et al. 2015). There is also evidence that edelfosine is active against embryos and eggs of *Caenorhabditis elegans* (Sanchez-Blanco et al. 2014). Furthermore, edelfosine is active against protozoa such as *Leishmania major*, *L panamensis* and *L. braziliensis* in infected macrophage in culture and in experimental infections in mice and hamsters (Varela et al. 2012). In *S. venezuelensis* infection, faecal eggs are found on day 5 p.i peaking on days 7-8 p.i and are cleared by 20 days p.i allowing to assess new drugs in a short time. Moreover is a synchronous infection permitting to study the effect on each parasitic phase. Edelfosine needs around 5 days to reach therapeutical concentration (Yepes et al. 2014) thus its administration had to start at the time of infection to reach therapeutical concentrations at the time when most of the parasites are adults. Further studies should be carried out to address the use of edelfosine as an alternative or preventive treatment.

In addition, induction of apoptosis-like cell death was observed in *S. venezuelensis* larvae following edelfosine treatment as assessed by TUNEL assay. The data reported here indicate that edelfosine induces DNA strand breaks in *S. venezuelensis* larvae, likely through an apoptosis-like cell death mechanism involved in the antiparasitic action of edelfosine. This suggests a major role of an apoptosis-like response in the cell death mechanism but additional putative mechanisms cannot be ruled out. This is in agreement with the ability of edelfosine to promote

apoptosis or apoptosis-like cell death in a number of distinct cell targets, including cancer cells (Gajate and Mollinedo 2007) as well as *Leishmania* and *Schistosoma* parasites (Varela et al. 2012, Yepes et al. 2015) as visualized by TUNEL assays. It has also been observed that apoptosis could be an effective mechanism using nitric oxide donors in *S. venezuelensis* larvae and *in vitro* cultures of adults (Ruano et al. 2012). Activated macrophages and treatment with edelfosine could be synergistic against this nematode.

Since its introduction for the treatment of strongyloidiasis, ivermectin was considered the drug of choice for this purpose together with albendazole, mebendazole and thiabendazole. All these drugs have broad-spectrum anthelmintic effect and have been used widely against human and veterinary parasites. Benzimidazole and ivermectin resistant-nematode strains have also been reported in grazing livestock (von Samson- Himmelstjerna 2012, Shalaby 2013) and the issue of decreased susceptibility in human nematodes or the possibility of resistance is also increasing (Geerts and Gryseels 2000, Osei-Atweneboana et al. 2011, Vercruysse et al. 2011). Moreover, ivermectin treatment failure has also been observed in coinfected patients with *S. stercoralis* and human T- lymphotropic virus-1 (HTLV-1) and also adverse events linked to sensitive human genotypes (Carvalho and Da Fonseca Porto 2004, Bourguinat et al. 2010). These situations make the identification of alternative chemotherapies a high priority issue.

Our results indicate that edelfosine significantly reduces worm recovery and egg laying in experimental infections and kills L3 *in vitro* through an apoptotic-like cell death mechanism. The proapoptotic activity of edelfosine as a putative mechanism opens the possibility to combination of drugs that could promote a synergistic action or minimize the possibility of drug resistance (Panic et al. 2014). In addition, edelfosine offers

additional advantages as an antiparasitic drug, such as the activity against different tumoral cells (Mollinedo et al. 2010), and the anti-inflammatory activity shown by this APL together with its low toxicity profile (Mollinedo et al. 2009), could be of interest in treatment of strongyloidiasis patients coinfected with *Leishmania* or *Schistosoma* parasites (Yepes et al. 2014).

In conclusion, strongyloidicidal activity of the alkylphospholipid edelfosine against *S. venezuelensis* was demonstrated using L3 cultures and a strongyloidiasis murine model, possibly through induction of apoptosis-like cell death. It was found significant reductions in egg burden, and it might thus warrant further investigation in other in order to establish the advantage as a therapeutical drug in soil-transmitted helminthic infections.

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Competing interest: The authors declare that they have no competing interest.

Figure legends

Fig. 1. The chemical structure of the alkylphospholipids edelfosine, miltefosine and perifosine.

Fig. 2. *In vitro* effects of edelfosine, miltefosine and perifosine on the viability of *S. venezuelensis* third stage larvae by the XTT assay (mean ± SD) cultured for 72 h. ANOVA: edelfosine $F_{(7, 40)} = 294.0$, $P < 0.001$; miltefosine $F_{(7, 40)} = 99.3$, $P < 0.001$; perifosine $F_{(7, 40)} = 85.6$ $P < 0.001$ * Significant reduction compared to untreated control group post-hoc Tukey's honest significance test $P < 0.001$

Fig. 3. Faecal egg production (mean ± SD) in mice infected with third stage larvae treated with increasing concentrations of edelfosine. ANOVA: edelfosine $F_{(5, 29)} = 92.1$, $P < 0.001$; miltefosine $F_{(5, 29)} = 69.1$, $P < 0.001$; perifosine $F_{(5, 29)} = 49.5$ $P < 0.001$ * Significant reduction compared to untreated control group *post-hoc* Tukey's honest significance test $P < 0.05$.

Fig. 4. Box-plots plus means (+) showing effects of the treatment with edelfosine and ivervectin in mice infected with 3000 *S. venezuelensis* L3 on day 7 post-infection in (A) parasitic females recovered from the intestine and (B) faecal egg count. ANOVA: parasitic females $F_{(2, 12)} = 17.5$, $P < 0.001$; faecal egg count $F_{(2, 12)} = 16.7$, $P < 0.001$. *Significant reduction compared to untreated control group *post-hoc* Tukey's honest significance test $P < 0.01$.

Fig. 5. Faecal egg emission (mean ±SD) in mice infected with 3000 *S. venezuelensis* and treated with edelfosine and ivermectin on days 5, 9, 11, 15, 17 and 19 post-infection. ANOVA: day 9 p.i. $F_{(2, 15)} = 24.2$, $P < 0.001$; day 11 p.i. $F_{(2, 15)} = 3.7$, $P = 0.049$; day 15 p.i. $F_{(2, 15)} = 19.8$, $P < 0.001$; day 17 p.i. $F_{(2, 15)} = 16.6$, $P < 0.001$; day 19 p.i. $F_{(2, 15)} = 7.5$, $P = 0.005$. * Significant reduction in edelfosine treated mice compared to untreated control group *post-hoc* Tukey's honest significance test $P < 0.05$.

Figure 6. Assessing the apoptotic effect of edelfosine using the TUNEL assay. *S. venezuelensis*-L3 were treated with 100 µM edelfosine for 72 h, and then analysed by confocal microscopy for propidium iodide staining (PI), visualizing in red all the parasite cells (A), TUNEL assay, staining in green the cells with disrupted DNA, and differential interference contrast (DIC). Merged images of PI and TUNEL panels show the apoptotic nuclei in yellow. Data shown are representative of four experiments performed.

Appendix A. Supplementary data

Videos. The effect of alkylphospholipids edelfosine (videos S1-S3), miltefosine (videos S4-S6) and perifosine (videos S7-S9) on *S. venezuelensis* larvae was scored as change in the pattern of motility in videos. Mortality was attributed as any movement detected during 2 min of observation under the microscope (magnifications 4x) [1], at 24, 48 and 72 h. Untreated *S. venezuelensis* larvae and ivermectin-treated larvae are also shown in each supplementary video.

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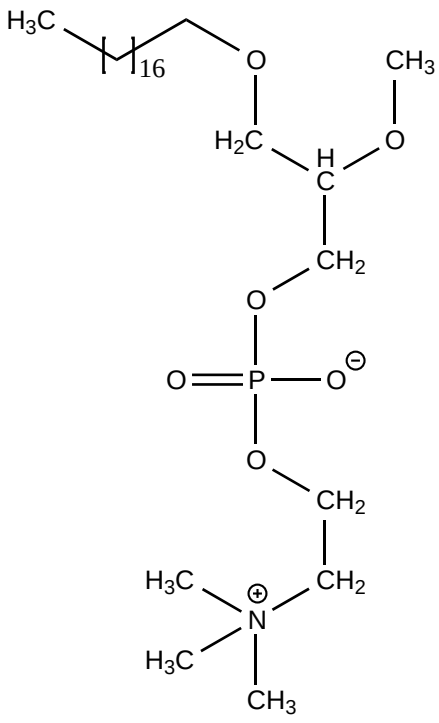
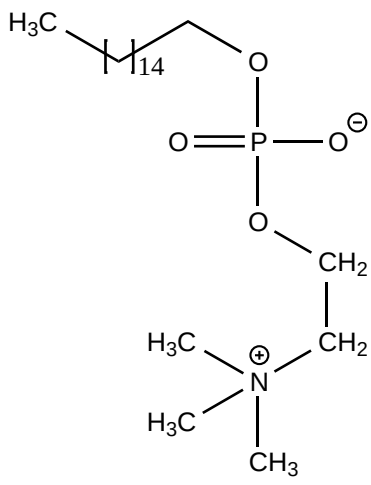
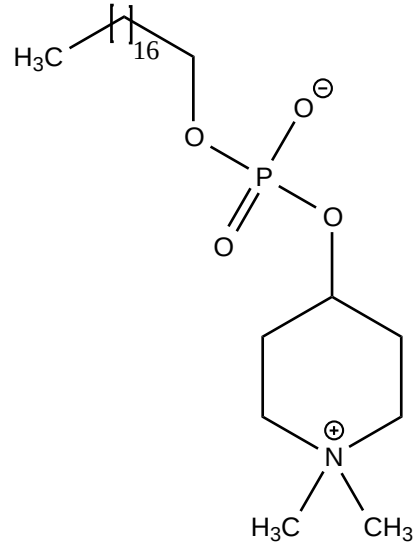
Edelfosine

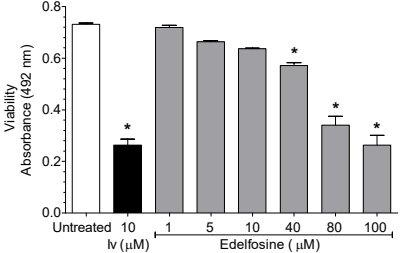


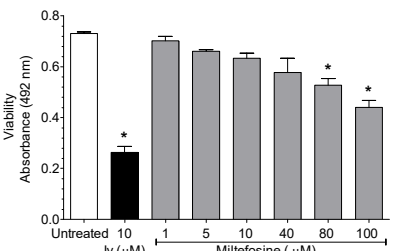
Miltefosine

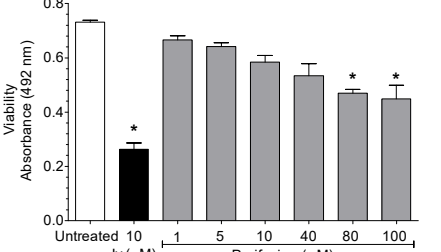


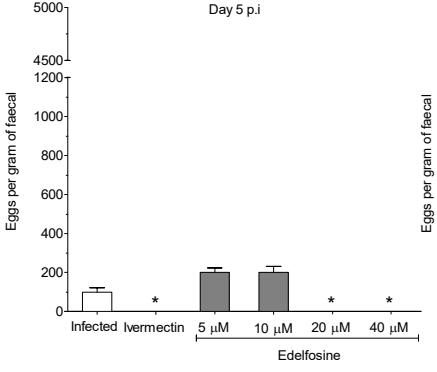
Perifosine

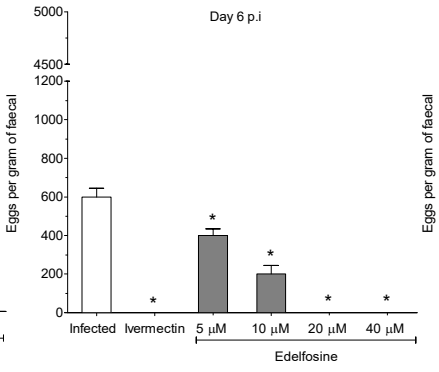


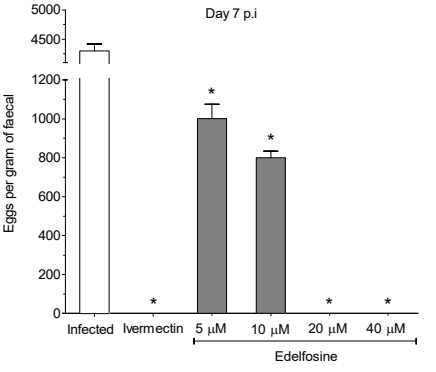

Viability
Absorbance (492 nm)
0.0
0.2
0.4
0.6
0.8
Untreated
10
Iv (μM)
1
5
10
40
80
100
Edelfosine (μM)

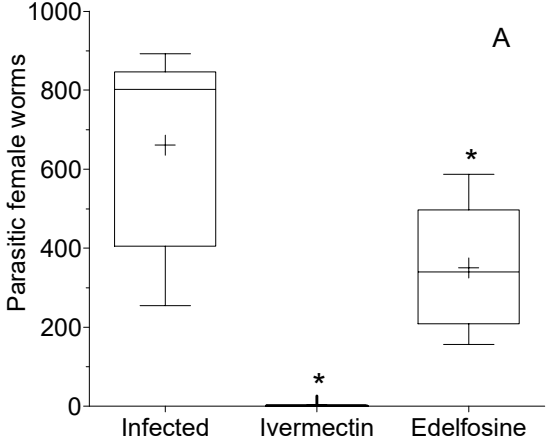

Viability
Absorbance (492 nm)
0.0
0.2
0.4
0.6
0.8
Untreated
10
Iv (μM)
1
5
10
40
80
100
Miltefosine (μM)

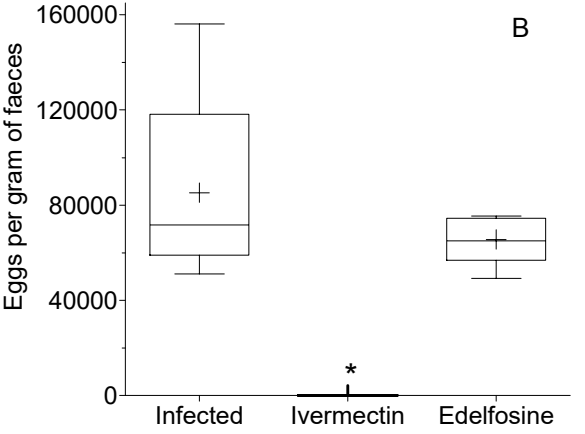

Viability
Absorbance (492 nm)
0.0
0.2
0.4
0.6
0.8
Untreated
10
Iv (μM)
1
5
10
40
80
100
Perifosine (μM)

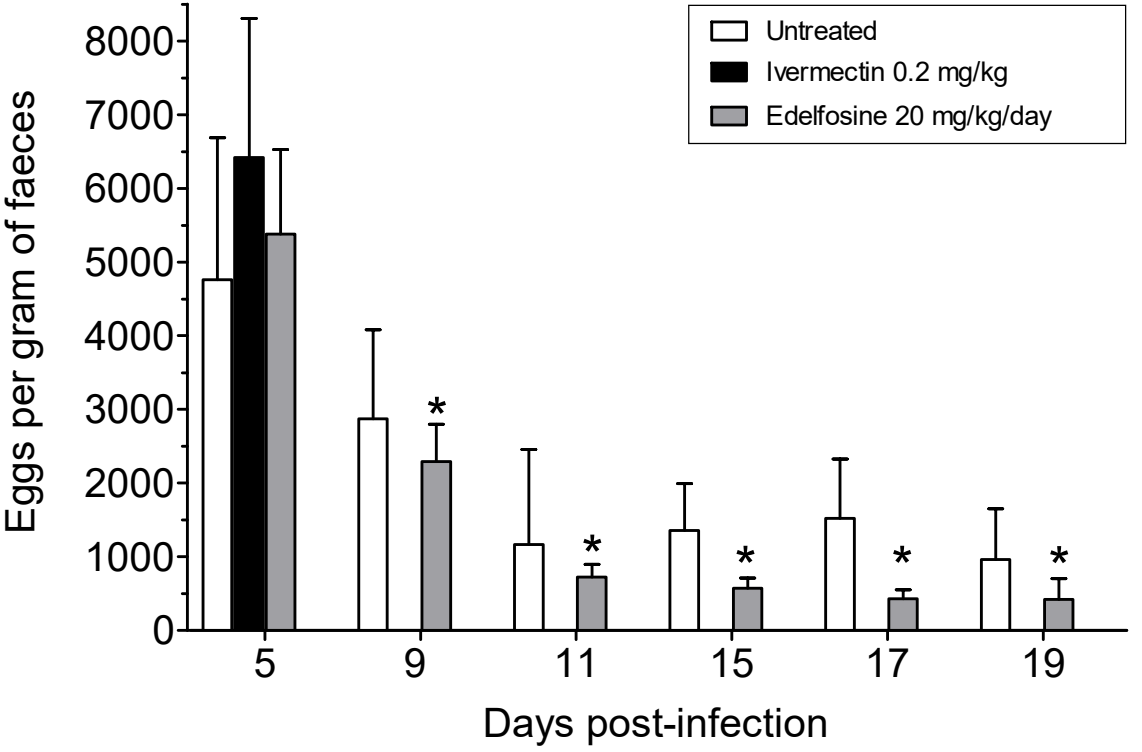

Day 5 p.i
Eggs per gram of faecal
5000
4500
1200
1000
800
600
400
200
0
*
Infected
Ivermectin
5 μM
10 μM
20 μM
40 μM
Edelfosine


Day 6 p.i
Eggs per gram of faecal
5000
4500
1200
1000
800
600
400
200
0
*
Infected
Ivermectin
5 μM
10 μM
20 μM
40 μM
Edelfosine


Day 7 p.i
Eggs per gram of faecal
5000
4500
1200
1000
800
600
400
200
0
*
Infected
Ivermectin
5 μM
10 μM
20 μM
40 μM
Edelfosine


1000
800
600
400
200
0
Parasitic female worms
A
*
*
Infected
Ivermectin
Edelfosine


160000
120000
80000
40000
0
Eggs per gram of faeces
B
*
Infected
Ivermectin
Edelfosine


Untreated
Ivermectin 0.2 mg/kg
Edelfosine 20 mg/kg/day
Eggs per gram of faeces
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
*
*
*
5
9
11
15
17
19
Days post-infection

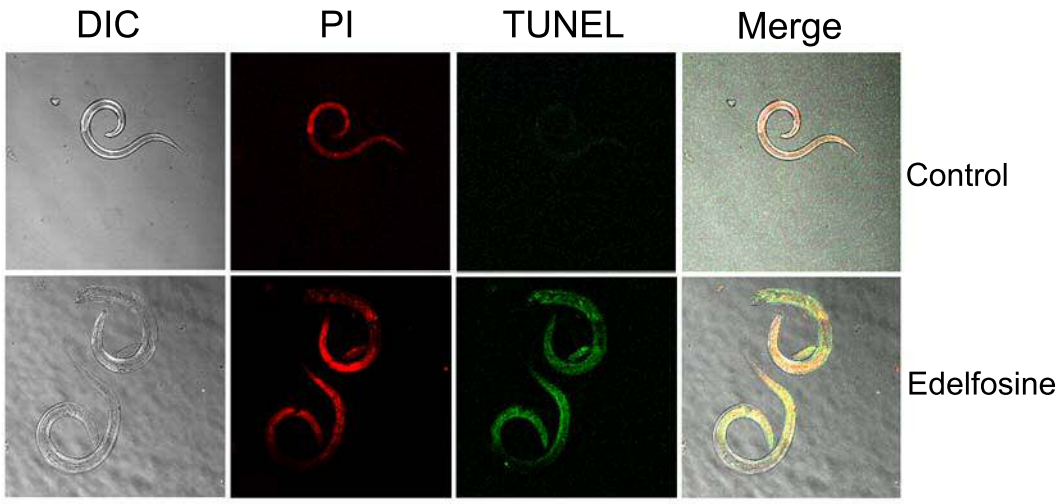

DIC
PI
TUNEL
Merge
Control
Edelfosine

Table 1. Cytotoxic effects of alkylphospholipids edelfosine, miltefosine and perifosine on mouse-derived macrophages cell line J774.2 using the XTT assay (mean ±standard deviation).

Concentration (μM)	Edelfosine (OD)	Miltefosine (OD)	Perifosine (OD)	Ivermectin (OD)
Untreated	1.699 ± 0.013	1.635± 0.023	1.681± 0.031	1.639± 0.025
1	1.551 ± 0.010	1.578 ± 0.068	1.575 ± 0.081	0.844 ± 0.038*
5	1.540 ± 0.022	1.557 ± 0.025	1.542 ± 0.023	0.747 ± 0.039*
10	1.474 ± 0.097	1.098 ± 0.338*	1.320 ± 0.225	0.662 ± 0.058*
40	0.930 ± 0.060*	0.765 ± 0.086*	0.922 ± 0.027*	0.575 ± 0.075*
80	0.792 ± 0.074*	0.694 ± 0.021*	0.864 ± 0.200 *	0.324 ± 0.002*
100	0.611 ± 0.021*	0.490 ± 0.053*	0.419 ± 0.035*	0.282 ± 0.047*
ANOVA	$F_{(5, 28)} = 162.6$ $P < 0.001$	$F_{(5, 28)} = 29.3$ $P < 0.001$	$F_{(5, 28)} = 36.8$ $P < 0.001$	$F_{(5, 28)} = 65.4$ $P < 0.001$

OD: Optical density at 492 nm. * Significant toxicity compared to untreated control group *post-hoc* Tukey's honest significance test $P < 0.001$.