

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

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21

22 **Abstract**

23

24 Strongyloidiasis is widely distributed in the tropical and subtropical areas.

25 Ivermectin is the drug of choice for the treatment. However, The concerns about relying
26 treatment on a single drug make identification of new molecules a priority.

27 Alkylphospholipid analogues, including edelfosine, are a group of synthetic compounds
28 that have shown activity against protozoan parasites and also against the helminth
29 parasite *Schistosoma mansoni*. The activity of edelfosine, miltefosine, perifosine against

30 *Strongyloides venezuelensis* was assessed both in cultures of third-stage larvae (L3) and
31 infected mice. The induction of an apoptosis-like mechanism in larvae after treatment

32 was studied. Larval motility and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-
33 tetrazolium-5-carboxanilide) assay were used to evaluate antiparasitic drug efficacy in

34 L3 cultures as screening test. Edelfosine displayed the highest activity against L3 and the
35 best selectivity index ($LD_{50} = 49.6 \pm 5.4 \mu M$, SI = 1.1) compared to miltefosine or

36 perifosine activity. L3 after culture with edelfosine were not able to develop an
37 infection in CD1 mice. The oral treatment with edelfosine showed reduction of 47% in

38 parasitic females allocated in the gut. Moreover, DNA fragmentation was also
39 observed by TUNEL staining in edelfosine treated L3. These data suggest that

40 edelfosine could be an effective drug against strongyloidiasis, probably through
41 apoptosis-like cell death.

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43 | 1. Introduction

44 | Strongyloidiasis is caused by nematodes of the genus *Strongyloides* widely
45 | distributed in tropical and subtropical areas. There are about 52 species in the genus,
46 | but only *S. stercoralis* and *S. fuelleborni* infect humans. It is estimated that 30 to 100
47 | million people are infected worldwide (Bisoffi et al. 2013, Puthiyakunnon et al. 2014).
48 | Infective third-stage larvae (L3) penetrate the skin, migrate to the lungs, reaching the
49 | trachea, oesophagus and small intestine where became mature. Parthenogenetic females
50 | lay eggs that hatch into rhabditiform larvae (L1), which are eliminated in the faeces.
51 | Some L1 remain and molt into L3 establishing reinfection cycles. The clinical
52 | strongyloidiasis ranges from asymptomatic infection, cutaneous *larva migrans*, Löeffler
53 | syndrome, chronic intestinal infection to life-threatening disseminated hyperinfection
54 | depending upon the immune status of the patient and the presence of risk factors,
55 | such as corticosteroid therapy, stem-cell transplantation, alcoholism or HTLV-1
56 | infection. Disseminated hyperinfection involves a massive spread of the parasite in
57 | situations of immunosuppression with high mortality (Montes et al. 2010, Schar et al.
58 | 2013, Sharifdini et al. 2014).

59 | Thiabendazole was long the drug of choice for treating strongyloidiasis.
60 | However, it is no longer available due to the strong adverse side effects. Albendazole,
61 | another broad- spectrum drug and anthelmintic agent is effective against *S. stercoralis*.
62 | Currently, ivermectin is the best therapeutic option for the treatment of strongyloidiasis,
63 | with cure rates from 70% to 85% of chronically infected patients (Pitisuttithum et al.
64 | 1995, Igual-Adell et al. 2004, Stuart et al. 2009). The anti-*Strongyloides* activity of
65 | ivermectin is superior to that of albendazole (Suputtamongkol et al. 2011).
66 | Alkylphospholipid (APL) analogues include edelfosine, miltefosine and perifosine, a
67 | heterogeneous group of natural lipids (Fig. 1) with promising anticancer activity. These

68 compounds act at the level of cell membranes affecting apoptotic signalling. Edelfosine
69 (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) is considered the prototype
70 APL molecule and a promising antitumor ether phospholipid drug that acts by activating
71 apoptosis through its interaction with cell membranes (Gajate and Mollinedo 2002,
72 Mollinedo et al. 2004, Gajate and Mollinedo 2007). In addition to its antitumor activity,
73 edelfosine exerts cytotoxic activity against parasitic protozoa such as *Leishmania* spp
74 (Varela et al. 2012), *Trypanosoma cruzi* (Luna et al. 2009), *Trichomonas vaginalis*
75 (Rocha et al. 2014), *Giardia lamblia* (Eissa and Amer 2012), *Acanthamoeba keratitis*
76 (Polat et al. 2012), *Neospora caninum* (Debache and Hemphill 2012), *Babesia* spp and
77 *Theileria equi* (AbouLaila et al. 2014). These compounds are also active against larval
78 stages and eggs of the free-living nematode *Caenorhabditis elegans* (Sanchez-Blanco et
79 al. 2014) and the blood fluke *Schistosoma mansoni* (Eissa et al. 2011, Bertao et al.
80 2012, Yepes et al. 2014). The human parasite *S. stercoralis* cannot complete its
81 development in immunocompetent mice and rats. Thus, attention has been focused on
82 the related parasites *Strongyloides ratti* and *Strongyloides venezuelensis*, parasite of
83 rats, to study host-parasite relationships (Sato and Toma 1990), and as general
84 experimental models of intestinal parasitism (Yasuda et al. 2014).

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

90

91 | **2 Materials and Methods**

92 | **2.1. Ethics statement**

93 The animal procedures in this study complied with the Spanish (L 6/3013, RD
94 53/2013) and European Union (Di 2010/63/CE) regulations regarding animal
95 experimentation for the protection and humane use of laboratory animals. The
96 University of Salamanca's accredited Animal Experimentation Facilities (Registration
97 number PAE/SA/001) were used for these procedures. The University of Salamanca's
98 Ethics Committee also approved the procedures that were used in this study (Permit
99 Number: 8402). The animals' health and welfare status was monitored throughout the
100 experiments by a health surveillance program according to Federation of European
101 Laboratory Animal Science Associations (FELASA) guidelines and also by the
102 University of Salamanca's standardized protocols. All efforts were made to minimize
103 suffering.

104

105 | **2.2. Animals**

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

116

117 | **2.3. Drugs**

118 | Edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocoline) was
119 | obtained from R. Berchtold (Biochemisches Labor, Bern, Switzerland). Miltefosine
120 | (hexadecylphosphocholine) was from Calbiochem (Cambridge, MA). Perifosine
121 | (octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate) was from Zentaris (Frankfurt,
122 | Germany). Stock sterile solutions of the distinct APLs (2 mM) were prepared in RPMI-
123 | 1640 culture medium (Invitrogen, Carlsbad, CA), supplemented with 10% heat-
124 | inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 IU/mL penicillin, and
125 | 100 µg/mL streptomycin, as previously described (Mollinedo et al. 1997). Ivermectin
126 | was purchased from Sigma Aldrich and diluted in dimethyl sulfoxide (DMSO) at a final
127 | concentration of 10 µM.

128

129 | **2.4. *S. venezuelensis* life cycle maintenance and parasitological techniques**

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

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

148

149 **2.5. Cytotoxicity in murine macrophages**

150 _____ For cytotoxicity assays the mouse-derived peritoneal macrophage cell line
151 J774.2 (Sigma-Aldrich) was used. Cells were maintained at 37 °C with a 5% CO₂
152 atmosphere and cultured in plastic culture flasks with Dulbecco's Modified Eagle
153 Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (Sigma-
154 Aldrich), 2 mM glutamine (Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich) and
155 100 µg/mL streptomycin (Sigma-Aldrich). 200 µL of a suspension containing 2×10^5
156 macrophages/mL were added onto 96 well flat-bottom microplates (NUNC) and
157 allowed to incubate for 2 h. After, APL's were added at different concentrations
158 ranging from 1 to 100 µM for the next 48 h and 50 µL of XTT were added to each well
159 and incubated again for 24 h at 37 °C, 5% CO₂. Finally, 100 µL of DMSO
160 (Sigma-Aldrich) were added to each well and the absorbance was measured at 492 nm
161 using an ELISA-plate reader (Anthos 2010; Anthos Labtec Instruments, Wals, Austria).
162 Ivermectin-treated, untreated and DMSO-treated macrophages were used as controls.
163 Each concentration was assayed in triplicate in three independent experiments and the
164 IC₅₀ value for each APL was calculated by sigmoidal regression analysis (Gomez-Ayala
165 et al. 2010).

166

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 °C 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 (LD₅₀) and it
186 | was calculated by sigmoidal regression analysis (Gomez-Ayala et al. 2010). The
187 | Selectivity Index (SI = mammalian cell IC₅₀/ Larva LD₅₀) for each compound was
188 | calculated to compare the strongyloidicidal activity with its respective mammalian cell
189 | cytotoxicity.

190

191 **2.7. Viability of edelfosine-treated-L3 in mice**

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

200

201 **2.8. Edelfosine activity against *S. venezuelensis* infection in mice**

202 | _____Two different experiments were performed to assess the *in vivo* activity of
203 | edelfosine. In the first experiment, mice were randomly distributed in three experimental
204 | groups with 5 mice per group as follows: Infected control; Infected and treated with
205 | ivermectin; Infected and treated with edelfosine. Ivermectin was administered orally at
206 | 0.2 mg/kg/day once on day 5 p.i. and edelfosine was administered at 20 mg/kg/day
207 | from the day of the infection to day 5 p.i. since migrant larvae and adults are present in
208 | human strongyloidiasis. All animals were infected by subcutaneous injection with
209 | 3000 L3 of *S. venezuelensis* resuspended in PBS. Edelfosine was administered to
210 | reach the therapeutical concentration synchronically with ivermectin after day 5 p.i
211 | following previous studies (Yepes et al. 2014). Parasitic females were recovered from
212 | the gut and eggs in faeces were counted on day 7 p.i using groups of five animals. In
213 | the second experiment, mice were randomly distributed in three experimental groups
214 | with six mice per group with the same groups as above and we conducted faecal egg
215 | counts on days 5, 7, 9, 11, 15, 17 and 19 p.i.

216 **2.9. Assessment of apoptosis-like cell death by TUNEL assay**

217

218 DNA fragmentation, which usually occurring in apoptosis-like cell death was
219 analysed by the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end
220 labeling) technique as previously described (Gajate et al. 2009). Larvae were washed
221 with PBS, centrifuged at 1500 rpm for 3 min, mounted on poly-L-lysine-coated
222 microscope slides (Menzel-Gläser, Braunschweig, Germany) and incubated for 24 h at
223 37 °C to stick the L3 to the slides. Larvae were then fixed in 4% paraformaldehyde
224 (Sigma, St. Louis, MO) for 25 min, and washed with PBS as above. Fixed parasites
225 were treated with trypsin and 0.25% EDTA, and stained for fragmented DNA using the
226 Fluorescein Apoptosis Detection System (Promega, Madison, WI) according to the
227 manufacturer's instructions. Propidium iodide was added for 15 min to stain nuclei of
228 both apoptotic and non-apoptotic cells in red, whereas fluorescein-12-dUTP was
229 incorporated at the 3'- OH ends of fragmented DNA resulting in localized green
230 fluorescence within the nuclei of apoptotic cells. Samples were analysed with a Zeiss
231 LSM 510 laser scan confocal microscope.

232

233 **2.10. Statistical analysis**

234 Data are expressed as mean and standard deviation (SD). Test for normality was
235 performed by Kolmogorov-Smirnov and homogeneity of variance was tested by the
236 Barrett test. Then one-way ANOVA analysis of variance, followed by Tukey's honest
237 significance (HDS) test was performed to determine any statistical differences between
238 treated and untreated controls. Differences were considered statistically significant at p
239 <0.05 . The data were processed using GraphPad Prism 5 (GraphPad Software, San
240 Diego, CA) for Mac.

241 3. Results

242 3.1. Experimental infection and treatments

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

251

252 3.2. Cytotoxicity in murine macrophages

253 | _____ Cytotoxicity of each compound was evaluated the in macrophage cell line by
254 | calculating the IC₅₀ for each APL, at six different concentrations. APL cytotoxicity
255 | was ranked as follow: edelfosine (IC₅₀ 53.4 ± 5.3 μM) < perifosine (IC₅₀ 52.8 ± 6.3 μM)
256 | < miltefosine (IC₅₀ 27.3 ± 3.8 μM), thus showing that edelfosine is the less toxic
257 | compound to the macrophages (Table 1).

258

259 3.3. In vitro activity of APLs against *S. venezuelensis* third stage larvae

260 | _____ First, *in vitro* the anti-*Strongyloides* activity of edelfosine, miltefosine and
261 | perifosine were analysed through XTT assay after 72 h of incubation using L3
262 | cultures with a range of 1-100 μM. Edelfosine showed the highest efficacy in killing
263 | the parasite, being effective at ≥ 40 μM with a LD₅₀ value of 49.6 ± 5.3 μM evaluated
264 | by the XTT assay (Fig. 2). It would be necessary to administer edelfosine 80 or 100
265 | μM to attain efficacy similar activity to that of ivermectin, the drug of choice for

266 | treating strongyloidiasis (LC50 = 0.41 ± 0.12 μM). Miltefosine and perifosine also
267 | showed efficacy in killing the parasite at higher concentrations, but their anti-
268 | *Strongyloides* activity seems rather poor as compared to either edelfosine or ivermectin
269 | (LD₅₀ = 85.7 ± 8.7 μM and LD₅₀ = 90.6 ± 10.5 μM, respectively; Fig. 2). Edelfosine
270 | also showed the highest Selectivity Index as demonstrated by the SI value, ranked as
271 | follows: edelfosine (SI 1.1) > perifosine (SI 0.6) > miltefosine (SI, 0.6).

272

273 | **3.4. Dose- and time-dependent effects of APLs on *S. venezuelensis* larvae**

274 | How the dose and the time of action of APL derivatives affected the parasites were
275 | investigated and microscopic observation of larvae incubated was performed at 24, 48
276 | and 72 h to determine decrease in motor activity. As shown in S1-S3 videos,
277 | edelfosine's activity is time and dose-dependent, reaching its maximum efficacy at 72 h
278 | showing mortality of *S. venezuelensis* L3 equal to 92% at 80 μM and 100% at 100 μM.
279 | These results were similar to those obtained with ivermectin at 10 μM. At 48 h
280 | significant efficacy of edelfosine (about 50%) was also found with 80 and 100 μM
281 | (videos S1-S3). Miltefosine's activity was also time and dose dependent and it was
282 | inferior to that of edelfosine, showing its maximum activity at 100 μM being close to
283 | 54% of reduction in larvae motility (videos S4-S6). Perifosine was the ALP with the
284 | lowest activity against larvae. Only at higher concentrations (≥ 80 μM) and after 72h of
285 | treatment perifosine reached a significant reduction of larvae motility (66%) (videos
286 | S7-S9). Untreated *S. venezuelensis* larvae remained alive during the next 72h after
287 | being cultured, reaching a motility of around 92%, whilst ivermectin-treated larvae were
288 | completely killed at 10 μM after 24h of treatment.

289

290 | **3.5. Edelfosine treated-L3 are not able to infect mice.**

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

300 | **3.6. Edelfosine *in vivo* activity in mice infected by *S. venezuelensis***

301 |
302 | _____ CD1 mice were subcutaneously infected with 3000 *S. venezuelensis* larvae and
303 | then orally treated with edelfosine during five consecutive days in order to reach a
304 | therapeutic level of the drug after day 5 p.i. when larvae are finishing their migration
305 | and reaching maturity and first eggs appear in faeces. In the first experiment, a
306 | significant reduction of 47% was observed in parthenogenetic females recovered from
307 | small intestines of edelfosine-treated mice on day 7 p.i. (350 \pm 162 females) compared
308 | to infected controls (661 \pm 259 females). A non-significant reduction in faecal egg
309 | counts (23 %) was observed on day 7 p.i. (65577 \pm 10402 EPG *cf* 85171 \pm 41012 EPG;
310 | Fig. 4). In the second experiment to assess the efficacy on egg laying curves significant
311 | reductions of faecal egg counts were observed from day 9 to the end of the
312 | experiment (Fig. 5).

314 3.7. Assessment of apoptosis-like cell death by TUNEL assay

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

326

327 4. Discussion

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

339 primary drug for treatment of strongyloidiasis, our data suggests that oral treatment
340 with edelfosine decreases significantly both the number of eggs per gram of faeces and
341 the number of parasitic female worms in the gut of mice. This efficacy is in
342 concordance with the schistomicidal activity against adults *in vitro* and *in vivo* and
343 the reduction of tissue egg burden in mice treated orally with edelfosine (Yepes et al.
344 2014). Moreover, a combination of edelfosine and praziquantel has demonstrated
345 efficacy against schistosomula (Yepes et al. 2015). There is also evidence that
346 edelfosine is active against embryos and eggs of *Caenorhabditis elegans* (Sanchez-
347 Blanco et al. 2014). Furthermore, edelfosine is active against protozoa such as
348 *Leishmania major*, *L. panamensis* and *L. braziliensis* in infected macrophage in culture
349 and in experimental infections in mice and hamsters (Varela et al. 2012). In
350 | *S. venezuelensis* infection, faecal eggs are found on day 5 p.i peaking on days 7-8 p.i
351 | and are cleared by 20 days p.i allowing to assess new drugs in a short time. Moreover is
352 | a synchronous infection permitting to study the effect on each parasitic phase.
353 | Edelfosine needs around 5 days to reach therapeutical concentration (Yepes et al.
354 | 2014) thus its administration had to start at the time of infection to reach therapeutical
355 | concentrations at the time when most of the parasites are adults. Further studies should
356 | be carried out to address the use of edelfosine as an alternative or preventive treatment.

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

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

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

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

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

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

400

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414 **Figure legends**

415

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

418

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

424

425 **Fig. 3.** Faecal egg production (mean \pm SD) in mice infected with third stage larvae
426 treated with increasing concentrations of edelfosine. ANOVA: edelfosine $F_{(5, 29)} = 92.1,$
427 $P < 0.001$; miltefosine $F_{(5, 29)} = 69.1, P < 0.001$; perifosine $F_{(5, 29)} = 49.5 P < 0.001$ *
428 Significant reduction compared to untreated control group post-hoc Tukey's honest
429 significance test $P < 0.05$.

430

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

437

438

439

440 **Fig. 5.** Faecal egg emission (mean \pm SD) in mice infected with 3000 *S. venezuelensis*
441 and treated with edelfosine and ivermectin on days 5, 9, 11, 15, 17 and 19 post-
442 infection. ANOVA: day 9 p.i. $F_{(2, 15)} = 24.2, P < 0.001$; day 11 p.i. $F_{(2, 15)} = 3.7, P =$
443 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
444 p.i. $F_{(2, 15)} = 7.5, P = 0.005$. * Significant reduction in edelfosine treated mice compared
445 to untreated control group *post-hoc* Tukey's honest significance test $P < 0.05$.

446

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

454

455 **Appendix A. Supplementary data**

456 Videos. The effect of alkylphospholipids edelfosine (videos S1-S3), miltefosine (videos
457 S4-S6) and perifosine (videos S7-S9) on *S. venezuelensis* larvae was scored as change
458 in the pattern of motility in videos. Mortality was attributed as any movement detected
459 during 2 min of observation under the microscope (magnifications 4x) [1], at 24, 48 and
460 72 h. Untreated *S. venezuelensis* larvae and ivermectin-treated larvae are also shown in
461 each supplementary video.

462

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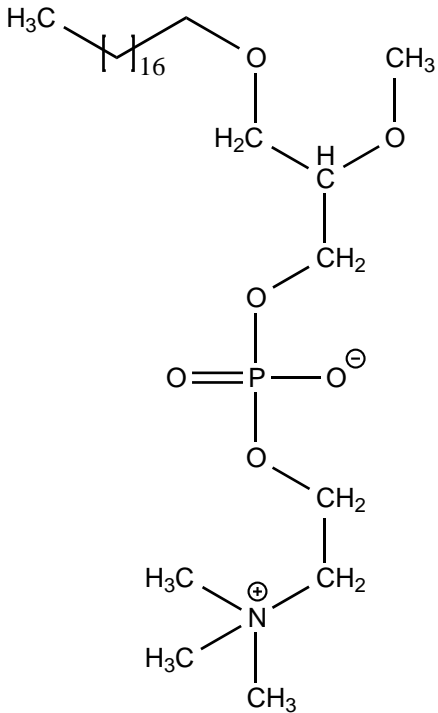
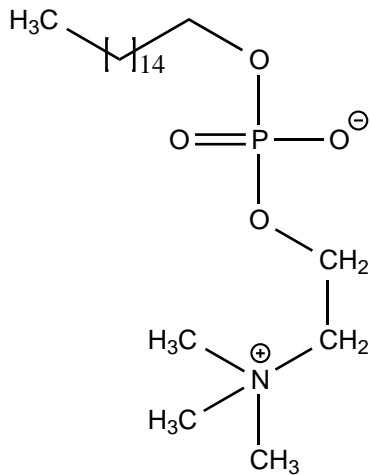
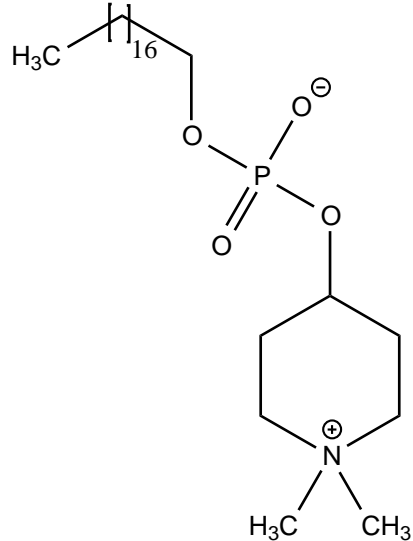
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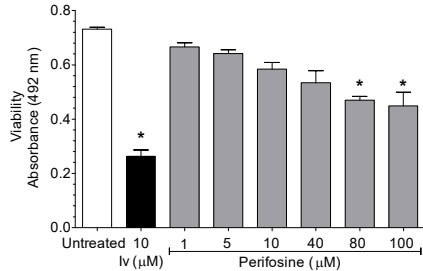
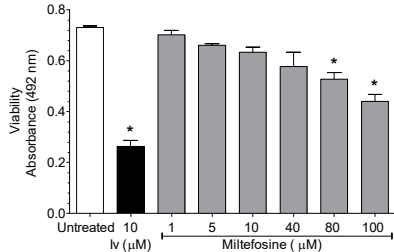
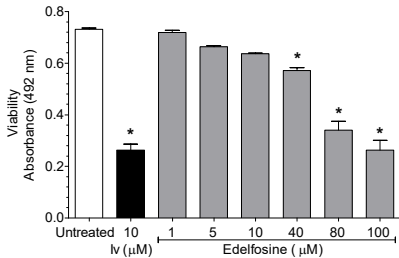
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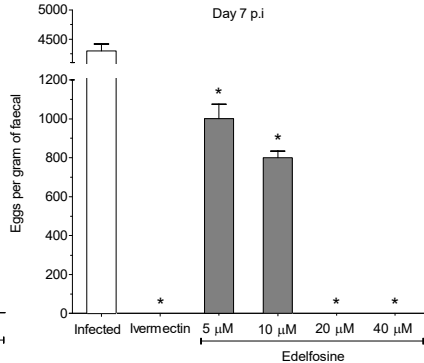
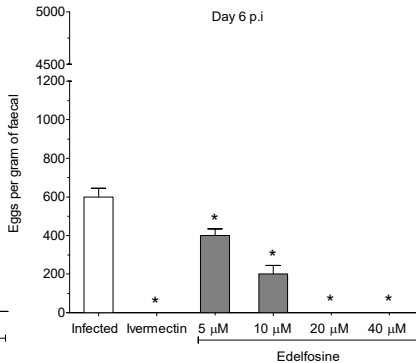
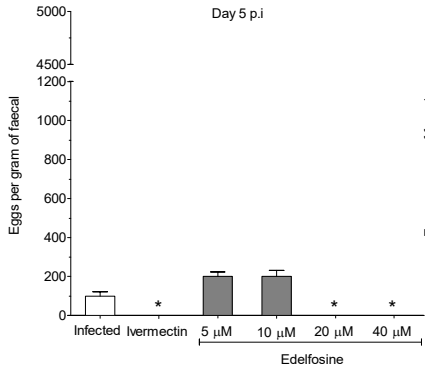
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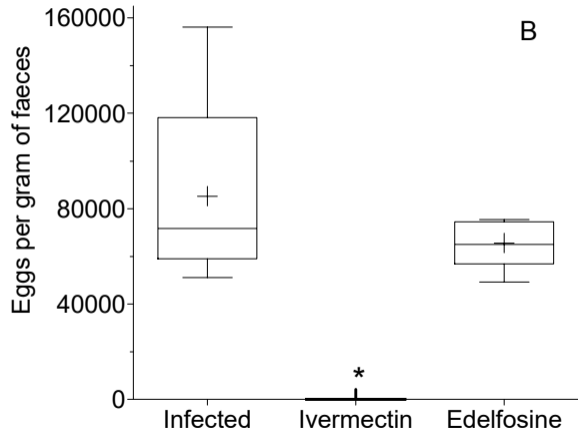
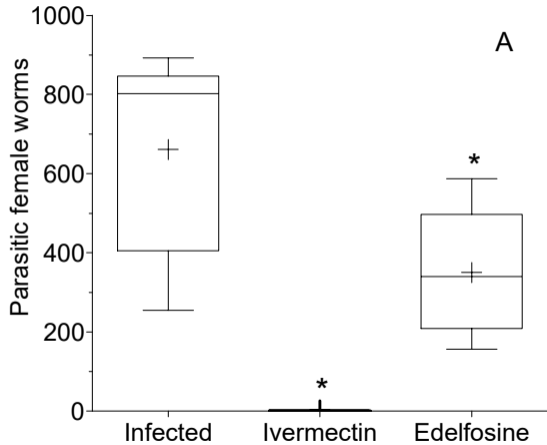
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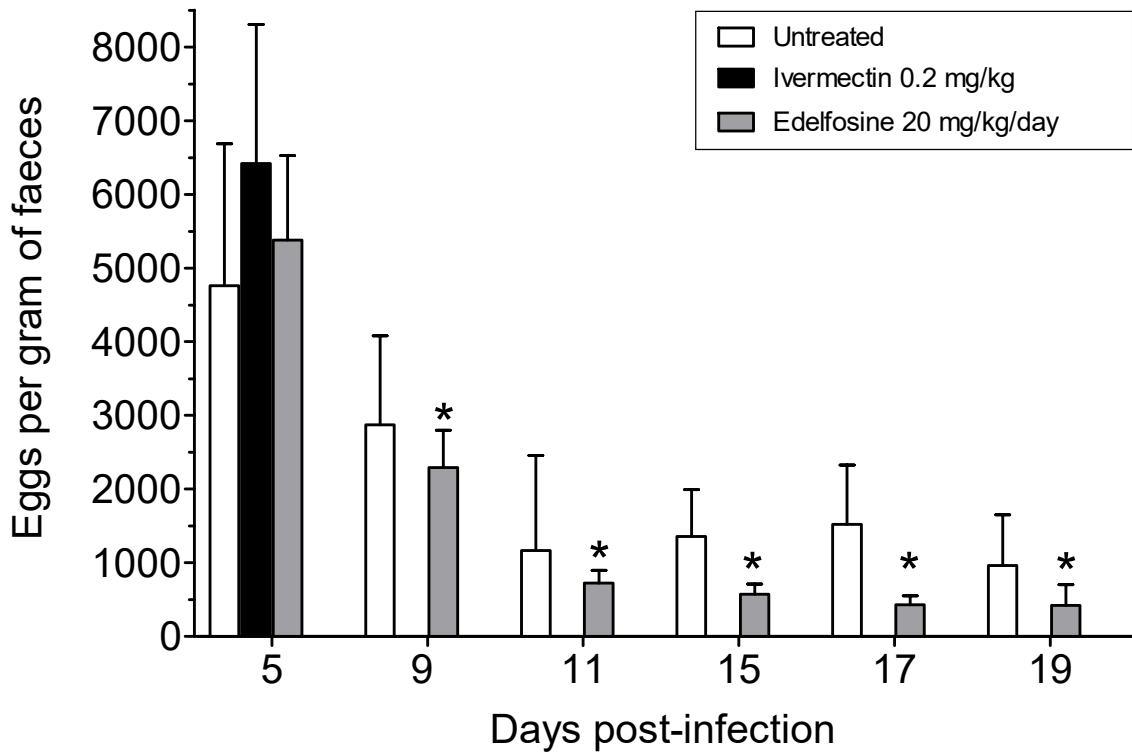
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Edelfosine**Miltefosine****Perifosine**









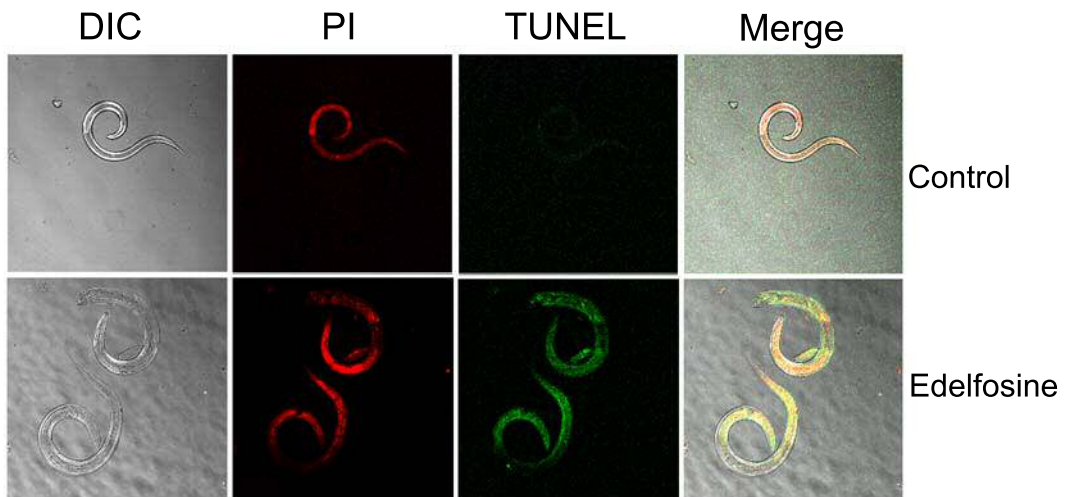


Table 1. Cytotoxic effects of alkylphospholipids edelfosine, miltefosine and perifosine on mouse-derived macrophages cell line J774.2 using the XTT assay (mean \pm standard deviation).

Concentration (μ M)	Edelfosine (OD)	Miltefosine (OD)	Perifosine (OD)	Ivermectin (OD)
Untreated	1.699 \pm 0.013	1.635 \pm 0.023	1.681 \pm 0.031	1.639 \pm 0.025
1	1.551 \pm 0.010	1.578 \pm 0.068	1.575 \pm 0.081	0.844 \pm 0.038*
5	1.540 \pm 0.022	1.557 \pm 0.025	1.542 \pm 0.023	0.747 \pm 0.039*
10	1.474 \pm 0.097	1.098 \pm 0.338*	1.320 \pm 0.225	0.662 \pm 0.058*
40	0.930 \pm 0.060*	0.765 \pm 0.086*	0.922 \pm 0.027*	0.575 \pm 0.075*
80	0.792 \pm 0.074*	0.694 \pm 0.021*	0.864 \pm 0.200*	0.324 \pm 0.002*
100	0.611 \pm 0.021*	0.490 \pm 0.053*	0.419 \pm 0.035*	0.282 \pm 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 $P < 0.001$.