(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 January 2017 (12.01.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/005774 A2

(51) International Patent Classification:
A61K 31/685 (2006.01)

(21) International Application Number:
PCT/EP2016/065943

(22) International Filing Date:
6 July 2016 (06.07.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PCT/EP2015/065383 6 July 2015 (06.07.2015) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

WO 2017/005774 A2

(54) Title: USE OF OLEYLPHOSPHOCHOLINE (OLPC) FOR PEVENTING OR TREATING CRYPTOSPORIDIOSIS

(57) Abstract: The present invention relates to the use of an alkylphosphocholine for the prevention or treatment of cryptosporidiosis. Specifically, the present invention relates to oleylphosphocholine (OIPC) for use in the prevention or treatment of cryptosporidiosis in a vertebrate, preferably a human vertebrate, and especially cryptosporidiosis wherein the causative agent is the oocysts Cryptosporidium parvum.

USE OF OLEYLPHOSPHOCHOLINE (OLPC) FOR PEVENTING OR TREATING CRYPTOSPORIDIOSIS

Description

The present invention relates to the use of an alkylphosphocholine for the prevention or treatment of cryptosporidiosis.

Cryptosporidium parvum is an obligate parasite of the phylum Apicomplexa that infects the microvilli of the small intestine of many mammalian hosts including humans. Considered as a major waterborne pathogen (drinking and recreational water) that can also be transmitted through contaminated foods, *C. parvum* outbreaks have been widely reported in numerous countries from all continents. This parasite can remain infectious for months in a humid environment. As a result, *C. parvum* has been categorized as a class B bioterrorist pathogen by the Center for Disease Control and Prevention (CDC).

As one of the major causative agents of cryptosporidiosis in humans, along with *Cryptosporidium hominis*, *C. parvum* causes mild self-limiting watery diarrhea or persistent and severe diarrhea depending on the age and the immune status of the patient. Indeed, *C. parvum* infection has been reported to be life-threatening in AIDS patients, among which the prevalence of cryptosporidiosis was determined to be 14% in developed countries and 24% in developing countries. *Cryptosporidium spp.,* along with *Giardia spp.,* has also been identified as the leading cause of chronic or persistent diarrhea in children in a context of malnutrition or immunodeficiency. Studies have shown that *Cryptosporidium* infections in young children have often resulted in stunting and lead to poor cognitive functions later in childhood. However, currently available treatments have demonstrated limited effect in these vulnerable populations.

Nitazoxanide, the only American Food and Drug Association (FDA) approved drug to treat cryptosporidiosis in immunocompetent patients, has shown little activity to fight against *C. parvum* infections in AIDS patients. Similarly, paromomycin, the currently used drug to treat *C. parvum* infections in AIDS patients, has shown modest activity and limited results in different case studies. With no efficient way to treat immunocompromised patients, many drugs have been tested over the years, but very few have shown consistent activity.

Oleylphosphocholine (OlPC, $C_{23}H_{48}NO_4P$) belongs to the family of alkylphosphocholines and is structurally related to miltefosine, which chemical formula is $C_{21}H_{46}NO_4P$. Both compounds have shown *in vitro* and *in vivo* anti-leishmanial activity.

Considering the above, it is an object of the present invention, amongst other objects to provide means for preventing and/or treating cryptosporidiosis and especially treating preventing and/or treating cryptosporidiosis caused by a *Cryptosporidium parvum* infection.

The above object, amongst other objects, is met by the present invention as outlined in the appended claims.

Specifically, the above object, amongst other objects, is met by the present invention, according to a first aspect, by oleylphosphocholine (OlPC) for use in the prevention or treatment of cryptosporidiosis in a vertebrate, preferably a human vertebrate, and especially cryptosporidiosis wherein the causative agent is the oocysts *Cryptosporidium parvum*.

According to an especially preferred embodiment, the present prevention or treatment comprises oral administration of oleylphosphocholine (OlPC).

According to a second aspect, the present invention relates to methods for preventing or treating cryptosporidiosis in a vertebrate, preferably a human vertebrate, comprising administering a therapeutically effective dose of oleylphosphocholine (OlPC) to said vertebrate. Preferably, the present administration is oral administration of said oleylphosphocholine (OlPC).

According to an especially preferred embodiment, the causative agent of cryptosporidiosis is *Cryptosporidium parvum*.

The present invention will be further detailed in the example below of especially preferred embodiments of the present invention. In the example, reference is made to figures wherein:

Figure 1: shows the in vitro efficacy of OlPC and miltefosine**.** (**A**) Parasite burden reduction in HCT-8 cells after 48 hours incubation with different concentrations of OlPC. (**B**) Parasite burden reduction in HCT-8 cells after 48 hours incubation with different concentrations of miltefosine. Error bars were calculated as ± S.E.M. (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ and **** = $P < 0.0001$).

Figure 2: shows survival curves in C57BL/6 IFNγR-KO mice infected with *C. parvum*. Results are shown in a survival curve.. Results do not include mice sacrificed at Day 10 for disease progression analysis. On Day 30 remaining mice were sacrificed along with uninfected controls.

Figure 3: shows oocyst shedding in C57BL/6 IFNγR-KO mice infected with *C. parvum*. All stool samples were collected from individual mice within each group, processed and analysed by qPCR. Results were normalized to shed oocysts per grams of stool. Error bars were calculated as ± S.E.M. (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ and **** = $P < 0.0001$).

Figure 4: shows parasite and oocyst burden in C57BL/6 IFNγR-KO mice infected with *C. parvum.* All intestinal samples were analysed by qPCR represented by the clear bars and by flow cytometry represented by the checkered bars. qPCR results were normalized to parasites per gram of intestines and flow cytometry results were normalized to oocysts per gram of intestines. (**A**) Parasite and oocyst burden in mice at Day 10. (**B**) Parasite and oocyst burden in mice at Day 30. Error bars were calculated as ± S.E.M.

Figure 5: shows day 10 histological sections of the ileum of control C57BL/6 IFNγR-KO mice infected with *C. parvum*. Representative transverse sections of the ileum stained with H&E showing disease progression. (**A**) Ileum of mice treated with PBS. Formation of fibrin is noticeable (yellow arrow). (**B**) Ileum of mice treated with 100 mg/kg/day of paromomycin. (**C**) Ileum of mice treated with 10 mg/kg/day of miltefosine. **A**, **B** and **C** shows severe epithelial damage and abundance of oocysts. Each group is represented at 100x and 400x magnifications. Red arrows indicate *C. parvum* oocysts.

Figure 6: shows day 10 histological sections of the ileum of OlPC-treated C57BL/6 IFNγR-KO mice infected with *C. parvum*. Representative transverse sections of the ileum stained with H&E showing disease progression. (**A**) Ileum of mice treated with 20 mg/kg/day of OlPC shows significant epithelial damage and abundance of oocysts. (**B**) Ileum of mice treated with 30 mg/kg/day of OlPC. (**C**) Ileum of mice treated with 40 mg/kg/day of OlPC. Sections from **B** and **C** reveals mild gut epithelial damage and rare presence of oocysts. Each group is represented at 100x and 400x magnifications. Red arrows indicate *C. parvum* oocysts.

Figure 7: shows day 30 histological sections of the ileum of control and 20 mg/kg/day OlPC-treated C57BL/6 IFNγR-KO mice infected with *C. parvum.* Representative transverse sections of the ileum stained with H&E showing disease progression. (**A**) Ileum of mice treated with 10 mg/kg/day of miltefosine shows severe epithelial damage and abundance of oocysts. (**B**) Ileum of mice treated with 20 mg/kg/day of OlPC shows some epithelial damage and moderate level of oocysts. Each group is represented at 100x and 400x magnifications. Red arrows indicate *C. parvum* oocysts.

Figure 8: shows day 30 histological sections of the ileum of 30 mg/kg/day OlPC-treated and 40 mg/kg/day OlPC-treated C57BL/6 IFNγR-KO mice infected with *C. parvum.* Representative transverse sections of the ileum stained with H&E showing disease progression. (**A**) Ileum of mice treated with 30 mg/kg/day of OlPC. Section reveals no gut epithelial damage and an absence of oocysts. (**B**) Ileum of mice treated with 40 mg/kg/day of OlPC presents no sign of epithelial damage and an absence oocysts. Each group is represented at 100x and 400x magnifications.

EXAMPLE

Materials and methods

Parasites

C. parvum (Iowa strain) oocysts were maintained through a C57BL/6 IFNγR-KO mouse infection model previously described. Oocysts were purified following the reported technique and were stored in 2.5% (w/v) aqueous potassium dichromate ($K_2Cr_2O_7$, Sigma-Aldrich, Oakville, Ontario, Canada) at 4°C.

Test compound

For *in vitro* studies, OlPC was diluted to a stock solution of 11.5 mM in ddH_2O. Miltefosine (Sigma-Aldrich, Oakville, Ontario, Canada) was used as a control in the *in vitro* assays because of its previously reported activity against *C. parvum*. Paromomycin (Sigma-Aldrich) was also used as a control in the *in vitro* assays. The drugs were prepared fresh daily in phosphate-buffered saline (PBS) for animal studies.

Host cells

For *in vitro* studies, human colonic tumor cells (ileocecal adenocarcinoma) (HCT-8: ATCC CCL-244) were used. Cells were maintained in T225 flasks (BD bioscience, Mississauga, Ontario, Canada) using DMEM media (Wisent, St-Bruno, Quebec, Canada). For OlPC *in vitro* studies, media was supplemented with 10 µg/ml gentamycin, 10 µg/ml streptomycin, 100 U/ml penicillin, 10% heat-inactivated fetal bovine serum (FBS), and non-essential amino acids (NEAA) (Wisent). For the cytotoxicity assays, cells were grown in supplemented DMEM without phenol red and further supplemented with 4.5g/L D-glucose (Sigma-Aldrich, Oakville, Ontario, Canada)

and 110 mg/L sodium pyruvate (Sigma-Aldrich). For miltefosine and paromomycin *in vitro* studies, media was supplemented with 10 µg/ml gentamicin, 50 µg/ml streptomycin, 50 U/ml penicillin and 10% heat-inactivated fetal bovine serum (FBS).

Cytotoxicity of OlPC in HCT-8 cells

XTT assay (Sigma-Aldrich) was used, which relies on the ability of live cells to cleave XTT causing a colorimetric change, to quantify the possible cytotoxicity of OlPC in HCT-8 cells. For this assay, DMEM without phenol red was used. Initially, 5×10^4 HCT-8 cells in 200 µL of supplemented DMEM were seeded in a 96-well flat-bottom plates (BD Falcon, Franklin Lakes, New Jersey, USA) and incubated overnight (or until they reached ~80% confluency) at 37°C with 5% CO_2. Freshly prepared serial dilutions of OlPC diluted in supplemented DMEM were used and 50 µL of these solutions were added to the wells to obtain final OlPC concentrations ranging from 10 µM to 1000 µM (4.33 µg/mL to 433 µg/mL). Samples were run in quadruplicate and plates were incubated at 37°C with 5% CO_2 for 48h. Then, 50 µL of XTT (dissolved in DMEM) was added to each well and plates were re-incubated for 4h. Absorbance was measured at 450 nm using a plate reader (EL800 BioTek; Fisher, Nepean, Ontario, Canada).

C. parvum infection model in HCT-8 cells

The effect of OlPC was tested on *C. parvum* sporozoites in HCT-8 cells. Briefly, 24-well flat-bottom plates (BD Falcon) were seeded with 3×10^5 cells in 1.4 mL of supplemented DMEM and incubated overnight (or until they reached ~80% confluency) at 37°C with 5% CO_2. Stock oocysts in 2.5% $K_2Cr_2O_7$ were washed three times with 0.1M acetate-NaCl buffer, pH 5.5, and subsequently incubated in 10 mM sodium periodate on ice for 20 min. Oocysts were then washed three times with PBS containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich). To release the sporozoites from the oocysts (excystation), parasites were incubated in DMEM containing 0.75% sodium taurocholate (Sigma-Aldrich) at 37°C for 30 minutes or until 50% excystation was determined by microscopy. Then, sporozoites from 1×10^5 oocysts were inoculated in each well. Non-infected cells incubated with supplemented DMEM served as negative controls as well as infected cells incubated with supplemented DMEM, as positive controls. OlPC was serially diluted in supplemented DMEM and was subsequently added to each well at concentrations ranging from 10 pM to 100 µM (4.33 pg/mL to 43.3 µg/mL).

Miltefosine and paromomycin, diluted in supplemented DMEM, were also tested at concentrations ranging from 10 pM to 10 µM (4.08 pg/mL to 4.08 µg/mL) and 10 pM to 100 µM (7.14 pg/mL to 71.4 µg/mL) respectively, to serve as controls. Plates were further incubated for

48h at 37°C with 5% CO_2. *In vitro* assays were run two independent times and each condition was run in triplicate for the miltefosine assay and in duplicate for the OlPC and the paromomycin assays. At the completion of the incubation, supernatants were removed and cells were lifted using 0.25% trypsin in EDTA (Wisent). To assess parasite burden in each well and determine the efficacy of OlPC, miltefosine or paromomycin against *C. parvum*, DNA was extracted from samples using QIAamp DNA Mini Kit (Qiagen, Toronto, Ontario, Canada). Purified DNA was stored at -20°C until use.

Quantitative polymerase chain reaction primers, probe and standard curve

Parasite burden from DNA samples from *in vitro* assays as well as from mouse fecal and intestinal samples were assessed by quantitative polymerase chain reaction (qPCR) targeting the hsp70 gene of *C. parvum* (GenBank: U69698.2) using the LightCycler 2.0 system (Roche, Laval, Quebec, Canada). Primers and probe sequences (TaqMan) were used according to previously published sequences. Briefly, a master mix per sample was prepared by adding 5 µL of PCR-grade water (Roche), 2 µL of 10 µM of forward primer (CP_hsp70_fwd : 5'-AACTTTAgCTCCAgTTgAgAAAgTACTC-3'; Tib MolBiol, Adelphia, New Jersey, USA), 2µL of 10µM reverse primer (CP_hsp70_rvs: 5'-CATggCTCTTTACCgTTAAAgAATTCC-3'; MolBiol), 2 µL of 2 µM 5' 6-carboxyfluorescein reporter dye (FAM) probe with a 3' tetramethylrhodamine quencher dye (TMR) (hsp-taq: 5'-AATACgTgTAgAACCACCAACCAATACAACATC -3'; MolBiol), and 4 µL of buffer mix containing dNTP, $MgCl_2$ and Taq-polymerase (Roche). Prior to the polymerase chain reaction, 5 µL of sample and 15 µL of master mix were added to LightCycler capillaries (Roche). The initial denaturation step was conducted at 95°C for 15 minutes followed by 45 cycles of denaturation at 94°C for 15 seconds and annealing at 60°C for 1 minute (Shahiduzzaman et al., 2009).

In order to quantify parasite burden, a standard curve was necessary. First, *C. parvum* oocysts were diluted to concentrations of 1×10^1 to 1×10^6 in 200 µL and then, they were disrupted by five freeze-thaw cycles of 2 min incubation in liquid nitrogen followed by 2 min incubation in a 56°C water bath. Second, DNA from the oocyst extracts was purified using QIAamp DNA Blood Mini Kit (Qiagen) and these samples were run (qPCR) in the LightCycler 2.0; qPCR was used to correlate threshold cycle values (C_t values) to parasite burden (R^2=0.9989). This standard curve was used to extrapolate parasite burden from DNA samples from *in vitro* assays as well as from mouse stool and intestine samples.

Immunocompromised mouse model of C. parvum infection

C. parvum oocysts in 2.5% $K_2Cr_2O_7$ were washed three times with PBS; parasites were then counted using a hemocytometer and diluted in PBS to a concentration of 4×10^4/mL. Six to eight week old C57BL/6 IFNγR-KO female and male mice (Jackson Laboratories, Bar Harbor, Maine, USA) were infected with a lethal dose of 4000 oocysts in 100 µL of PBS on Day 0 using a 22-gauge gavage needle (CDVM, St-Hyacinthe, Quebec, Canada). Mice were separated in 8 groups (13 mice/group for treated groups and 4 to 8 mice/group for control groups). Groups 1 and 2 were non-infected controls, treated per os (oral gavage) with PBS or with 40 mg/kg/day of OlPC, respectively. Group 3 was infected and treated with PBS (positive controls). Groups 4, 5 and 6 were infected and treated per os with 40 mg/kg/day, 30 mg/kg/day and 20 mg/kg/day of OlPC, respectively. Groups 7 and 8 were infected controls treated per os with 10 mg/kg/day of miltefosine and 100 mg/kg/day of paromomycin, respectively. Treatment started at Day 3 and continued for 10 days. OlPC and control drugs were prepared fresh on a daily basis for the length of the treatment. Mice were weighted daily. Starting on Day 4, individual stool samples were collected every three to four days until study termination.

Five mice from each group were sacrificed at Day 10 (before daily treatment) to assess and compare disease progression. Other mice were maintained until study termination (Day 30) unless they were found dead or had to be sacrificed due to severe illness. Because of the lethality of this model, an objective mouse rating system based on symptom severity and behavioral changes was developed in accordance with the Animal Care Committee and the Canadian Council on Animal Care (CCAC) guidelines to determine when mice should be sacrificed due to illness. Upon death or euthanasia, the mouse ileum was preserved in formalin for histological purposes and the rest of the intestine (duodenum to rectum) was collected individually and chopped into 0.5 cm pieces into a 60 mL specimen container (ThermoFisher Scientific, Ottawa, Ontario, Canada) with 10 mL of 0.02% (v/v) Tween20 in PBS (one container per mouse). During necropsy, stool was also collected for oocyst purification.

After completion of the *in vivo* study, a constant volume of purified oocysts from intestinal samples of mice from each treatment groups was inoculated to naïve C57BL/6 IFNγR-KO mice to determine if the concentration of oocysts remaining in the intestines of treated mice was able to induce clinical signs of cryptosporidiosis in naïve mice.

All mouse studies were conducted at the Montreal General Hospital rodent animal facility site of the Research Institute of McGill University Health Center. All studies performed were approved and in accordance with the MGH Facility Animal Care Committee and also in accordance with the CCAC guidelines.

Oocyst purification from intestinal samples and oocyst burden analysis by qPCR

For each mouse intestine sample in 0.02% (v/v) Tween20 in PBS, 0.05 g of sputasol (dry mixture of 10% DTT, 76% NaCl, 2% KCl, 10% Na_2HPO_4 and 2% $KHPO_4$) was added. Oocyst purification was performed using a previously described method reported in literature. Briefly, each sample was homogenized for 2 minutes at 7000 rpm with the Polytron PT3000D homogenizer (Kinematica, Lucerne, Switzerland). Between each sample, the homogenizer was cleaned with bleach and ethanol to avoid cross-contamination. Samples were placed on a rotary mixer at low speeds for 90 minutes at room temperature. Samples were then transferred to 50 mL Falcon tubes (BD Falcon, Franklin Lakes, New Jersey, USA) and centrifuged at 2000 × *g* for 10 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 8 mL of 0.02% (v/v) Tween20 in ddH_2O and 2 mL of diethyl ether (Sigma-Aldrich, Oakville, Ontario, Canada). Samples were thoroughly vortexed and centrifuged at 2000 × *g* for 10 min at 4°C. The layers containing fatty cells and tissues as well as the supernatants were removed. The pellets were washed with 20 mL of cold ddH_2O and centrifuged at 2000 × *g* for 10 minutes at 4°C. Supernatants were discarded and the pellets resuspended in 20 mL of saturated NaCl. Samples were vortexed thoroughly, carefully overlayed with 5 mL of cold ddH_20 and centrifuged at 2000 × *g* for 10 min at 4°C. Five milliliters of the interphase were collected and centrifuged at 2000 × *g* for 10 min at 4°C; supernatants were discarded and oocyst-containing pellets were resuspended in 1 mL PBS containing 10 µg/ml streptomycin and 100 U/ml penicillin. Purified oocyst samples were stored at 4°C. For qPCR analysis, 200 µL were taken from each purified oocyst sample and five freeze/thaw cycles of 2 minutes incubation in liquid nitrogen followed by 2 minutes incubation in a 56°C water bath were performed. DNA was then extracted using the QIAamp DNA Blood Mini Kit (Qiagen) and samples was used for qPCR analysis.

Oocyst purification from stool samples and oocyst shedding analysis by qPCR

Individual stool samples were collected in 500 µL of distilled and deionized (18.2 MΩ·cm) water (ddH_2O) and stored in microcentrifuge tubes at 4°C until used. Samples were thoroughly vortexed until stools were homogenized to smaller particles, then centrifuged at 14000 × *g* and washed with cold ddH_2O. Supernatants were discarded and the pellets resuspended in 1 mL of saturated NaCl. Samples were vortexed thoroughly, carefully overlayed with 250 µL of cold ddH_2O and centrifuged at 1600 × *g* for 10 min at 4°C. All of the supernatant was collected and the sample was exposed to another NaCl-ddH_2O overlay. The supernatants collected from both overlays were centrifuged at 14 000 × *g* for 3 min at 4°C, combined into one tube and resuspended in 200 µL of ddH_2O. Purified oocysts were exposed to five freeze/thaw cycles as described above.

DNA was extracted using the QIAamp DNA Blood Mini Kit and stored at -20°C until oocyst shedding was quantified by qPCR. Values were normalized by gram of stool.

Oocyst burden analysis by flow cytometry

All oocyst samples purified from mouse intestine were incubated in a final concentration of 1% paraformaldehyde for 15-30 minutes prior to analysis. Briefly, 40 µl of sample and 50 µl of CountBright™ absolute counting beads (Invitrogen, Burlington, Ontario, Canada) were added to a paraformaldehyde solution for a final volume of 500ul. Oocysts within each sample were quantified by morphology (FSC-A and SSC-A) using BD LSRFortessa™ cell analyser (BD, Franklin Lakes, New Jersey, USA). Oocysts were initially identified with a FITC-labelled mouse IgG3 monoclonal antibody targeting a surface antigen (AbD Serotec, Raleigh, North Carolina, USA). Results demonstrated that 98% of the FITC positive events occupied a distinct population by size and complexity in the FSC-A and SSC-A channels.

Results generated by flow cytometry are based on unstained samples gating on this distinct oocyst population. Oocyst concentration per sample was calculated by determining the quantity of sample collected based on the number of CountBright™ bead events collected. Final oocyst numbers were normalized by gram of mouse intestines.

Histopathology

C. parvum is known to preferentially infect the jejunum and ileum, thus the ileum was collected for histopathology. Liver and spleen were also fixed in formalin, paraffin-embedded and cut to 4 µm thick sections. Sections were stained with hematoxylin and eosin to further assess disease progression by light microscopy.

Data analysis

All *in vitro* studies and qPCR data were analysed using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA). Graphs and P values were obtained using GraphPad Prism® version 6.0 (GraphPad software Inc., La Jolla, California, USA). Results represent mean ± Standard Error Mean (SEM). For OlPC and miltefosine EC_{50} calculations, the same software, GraphPad Prism® version 6.0, the nonlinear fit of EC_{50} shift and the "shared value for all data sets" constraint for Bottom and Top were used. Flow cytometry data was analysed using FlowJo v10 analytical software (Treestar Inc., San Carlos, California, USA).

Results

OlPC and miltefosine inhibit C. parvum infection in HCT-8 cells in vitro

To test the efficacy of OlPC to inhibit *C. parvum* infection *in vitro*, HCT-8 cells were used which allowed the parasite to replicate. Miltefosine was used as a control as its efficiency to inhibit *C. parvum* infection *in vitro* has already been described. However, the dose of 100 µM was not tested for miltefosine as cell toxicity has been reported at a concentration of 24.5 µM when cells are incubated for 45 hours. For the OlPC and the miltefosine assays, DNA was extracted after 48 hours of incubation with the compound. A standard curve was used to correlate threshold cycle values (C_t values) obtained by qPCR to parasite burden. Then, for each well, the percentage of parasite burden reduction was assessed according to this formula:

=[1-(parasite burden from tested well/parasite burden of positive control)]*100

Positive controls were infected cells incubated with supplemented media (no compound). **Figure 1** represents the mean of the parasite burden reduction of tested wells for each concentration of OlPC (A) and miltefosine (B) ± Standard Error Mean (SEM).

HCT-8 cells treated with 100 µM, 10 µM and 1 µM of OlPC showed the highest *C. parvum* inhibition with 87%, 84% and 85% reduction in parasite burden, respectively (**Figure 1A**). Therefore, these concentrations of OlPC are not statistically different than uninfected controls showing the clear potency of these treatments to inhibit *C. parvum* infection *in vitro*. There is a statistical difference ($P < 0.0001$) between OlPC treatment and uninfected controls starting at the concentration of 100 nM due to the lack of efficiency of these doses to inhibit *C. parvum* infection. Results demonstrated a dose-dependent reduction of *C. parvum* burden when treated with increasing concentrations of OlPC and a calculated EC_{50} of 18.84 nM. Cell toxicity of OlPC was not observed at concentrations of ≤ 50 µM using an XTT assay.

Miltefosine at concentrations of 10 µM and 1 µM showed the highest inhibitory effect with 85% and 69% reduction in parasite burden, respectively (**Figure 1B**). Similarly to the OlPC treatment, at these concentrations, there is no statistical difference between the miltefosine treatment and uninfected cells. So, OlPC is as potent as miltefosine at inhibiting *C. parvum* infection *in vitro* at a concentration of 10 µM (84% vs 85%), but OlPC better inhibits infection at a concentration of 1 µM (85% vs 69%). The calculated EC_{50} for miltefosine is 0.81 µM, which is slightly lower than the previously published value of 1.87 µM for this compound and this parasite, but within the same range. Batch differences in oocyst virulence due primarily to freshness are normal and can explain this type of variation. Concentrations of paromomycin ranging from 10 pM

to 100 µM were tested to determine the ability of this compound to inhibit *C. parvum* infection *in vitro*. However, no significant effect was observed which is in agreement with what has been published previously.

OlPC shows a dose-dependent effect on C. parvum-induced mortality in an immunodeficient mouse model

The C57BL/6 IFNγR-KO mouse model is an excellent model to assess the efficacy of compounds to treat acute *C. parvum* infection because of the high susceptibility of these knockout mice to this parasite. According to previous reports, mice infected with as low as 1500 oocysts had a mortality rate of 80% by Day 14. Therefore, in this example, mice were infected with 4000 oocysts, and survival, as well as parasite burden reduction, was used to differentiate an effective treatment from an inefficient treatment. To ensure the objectivity of the study, an external blinded examiner scored the mice reaching pre-defined critical clinical end-points according to a pre-established rating system.

As expected, infected controls started showing symptoms of illness as early as Day 5 and had a very high mortality rate at Day 14. For the PBS treated group, mice started showing signs of illness (such as hunched back, weight loss, dehydration, lethargy and watery stools) at Day 5 and all mice from this group died or were euthanized between Day 6 and Day 10 (**Figure 2**). Mice treated with 100 mg/kg/day of paromomycin for 10 days displayed clinical symptoms of cryptosporidiosis at Day 7 and a 60% mortality rate was observed at Day 9. Some paromomycin-treated mice did survive until Day 13 but, the Mantel-Cox or the Gehan-Breslow-Wilcoxon tests used to compare survival curves showed no significant difference with the PBS-treated group (**Figure 2**).

Mice treated with 10 mg/kg/day of miltefosine also showed signs of illness as early as Day 7 and a 50% mortality rate at Day 13 but, survivors remained alive until the end of the study (Day 30) (Fig. 2).This clear difference in survival rates with the PBS-treated group ($P < 0.001$) determined that a 10 mg/kg/day miltefosine treatment for 10 days significantly decrease mortality. Nevertheless, at Day 30, clinical signs of cryptosporidiosis were still visible in these mice such as 15% weight loss, hunched backs, dehydration and soft stools.

Mice treated with 40 mg/kg/day of OlPC displayed a 100% survival rate by the experimental endpoint (Day 30) ($P < 0.001$) (**Figure 2**). Additionally, the onset of symptoms began later (Day 9) than the PBS-treated controls; clinical signs were less severe and did not last as long in comparison with other groups. Furthermore, at Day 10, which is the peak of infection, weight loss (about 10% reduction from initial weight for the 40 mg/kg/day OlPC-treated group) was already significantly lower compared to any of the infected control groups (>15% of initial

weight, $P < 0.05$, data not shown). By Day 30, 40 mg/kg/day OlPC-treated mice looked healthy as they displayed no visible signs of illness. Moreover, unlike the miltefosine-treated group, 40 mg/kg/day OlPC-treated mice had exceeded, by Day 30, their initial weights ($P < 0.0001$.

Mice treated with 30 mg/kg/day of OlPC for 10 days, had less severe clinical signs of cryptosporidiosis. For these latter mice, on one hand, treatment was not able to rescue all mice which showed a severe weight loss of up to 15% of initial weight; on the other hand, the onset of symptoms was delayed to Day 8 or 9. Their survival rate was of 87.5% at Day 11, 75% at Day 30 (**Figure 2**) and, by Day 30, the survivors displayed no visible signs of illness and had returned to their initial weights (data not shown). Finally, the lowest dose of OlPC (20 mg/kg/day) was still able to rescue 75% of mice at Day 30 even if there were still visible signs of illness and mice had a 5% weight loss in comparison with initial weights. Non-infected mice treated with 40 mg/kg/day of OlPC presented a constant weight through the length of the study and no clinical sign were noted in this group.

OlPC eliminates oocyst shedding in a dose-dependent manner

All infected mice began shedding oocysts between Day 5 and Day 7. Stool samples were collected from individual mice, processed and analysed by qPCR. At Day 10, infected mice treated with PBS and with 100 mg/kg/day of paromomycin were shedding 8.52×10^7 and 1.84×10^8 oocysts per gram of stool (oo/g of stool) respectively. Similarly, mice treated with 10 mg/kg/day of miltefosine were shedding 1.94×10^8 oo/g of stool at Day 10 (**Figure 3**). At Day 14 and 30, surviving mice of this group continued to shed considerable levels of oocysts; 1.49×10^7 and 1.5×10^7 oo/g of stool respectively (**Figure 3**).

In mice treated with 40 mg/kg/day of OlPC, oocyst shedding reduction occurred as early as Day 10 with only 7.59×10^6 oo/g of stool which is a significant reduction in comparison with the 10 mg/kg miltefosine group ($P < 0.01$) (**Figure 3**). At Day 14, after 10 days of OlPC-treatment, oocyst shedding in this group was even lower with 5.14×10^3 oo/g, representing a 99.96% reduction (4-log reduction) in oocyst shedding. At Day 14, the 40 mg/kg/day OlPC-treated group is also statistically different when compared with the 10 mg/kg miltefosine-treated mice ($P < 0.05$) (**Figure 3**). Finally, at Day 30, there was a 100% elimination of oocyst shedding (>7-log reduction) in all mice of the 40 mg/kg/day OlPC-treated group ($P < 0.0001$) (**Figure 3**). Light microscopy was used to confirm qPCR results. Indeed, all the samples of 40 mg/kg/day OlPC-treated mice at Day 30 were negative as no oocysts could be visualized under the microscope.

Mice treated with 30 mg/kg/day of OlPC showed a similar tendency in oocyst shedding as the 40 mg/kg/day OlPC-treated group. Effectively, all average oocyst counts were significantly lower when compared with the 10 mg/kg/day miltefosine-treated group (**Figure 3**); at

Day 10, the average oocyst shedding was 2.45×10^6 oo/g of stool ($P < 0.0001$), at Day 14, 4.9×10^2 oo/g of stool ($P < 0.05$) and, at Day 30, 13 oo/g of stool ($P < 0.01$). Mice treated with 20 mg/kg/day of OlPC had the least important reduction in oocyst shedding but remained significantly lower than the 10 mg/kg miltefosine-treated mice. In fact, 4.04×10^7, 3.68×10^5 and 5.95×10^3 oo/g of stool, was evaluated in these 20 mg/kg/day OlPC-treated mice at Day 10 ($P < 0.01$), Day 14 ($P < 0.01$) and Day 30 ($P < 0.05$) respectively (***Figure 3***).

Oocyst shedding was not statistically different between mice treated with 30 mg/kg/day and 40 mg/kg/day at Day 10 and Day 14. The fact that the numerical value of the mean for parasite burden/g of stool for the 40 mg/kg/day treated mice was slightly higher at Day 10 and Day 14 than the one for 30 mg/kg/day treated mice is most probably due to inter-individual variations in this mouse model.

OlPC significantly reduces parasite burden in the intestines of C57BL/6 IFNγR-KO mice infected with C. parvum

To quantitate parasite and oocyst burden in the intestines and assess disease progression of infected mice during and after their respective treatments, mice were euthanized and intestines were collected at two time points: Day 10 or Day 30. Intestinal samples from every mouse were processed individually to purify *C. parvum* parasites, analysed using qPCR and normalized to parasites per gram of intestines (p/g of intestines). On Day 10, infected control groups revealed massive levels of *C. parvum* in the gut.

Mice treated with PBS had the highest parasite burden with 4.77×10^7 p/g of intestines followed by mice treated with 100 mg/kg/day of paromomycin with 5.01×10^6 p/g of intestines (**Figure 4A**). Mice treated with 10 mg/kg/day of miltefosine also had very high parasite burden with 7.94×10^6 p/g of intestines (**Figure 4A**). In mice treated with OlPC, parasite burden was significantly lower compared to the infected PBS control group. After seven days of treatment (Day 10), 20 mg/kg/day OlPC-treated mice showed a parasite burden of 3.44×10^6 p/g of intestines ($P < 0.01$) (**Figure 4A**). In mice treated with 30 mg/kg/day of OlPC the parasite burden was even lower with 1.81×10^5 p/g of intestines ($P < 0.001$) corresponding to a 99.6% (2-log reduction) parasite burden reduction in comparison with the PBS control group (**Figure 4A**). Similarly, mice treated with 40 mg/kg/day of OlPC also revealed a 99.6% reduction in parasite burden with 2.25×10^5 p/g of intestines ($P < 0.001$) when compared to the infected PBS control mice (**Figure 4A**).

By Day 30, 17 days after the completion of the treatments, surviving mice treated with 10 mg/kg miltefosine exhibited a high parasite burden with 2.65×10^6 p/g of intestines (**Figure 4B**). Parasite burden decreased in mice treated with OlPC when compared to the 10 mg/kg

miltefosine-treated mice following a dose-dependent response. Indeed, in mice treated with 20 mg/kg/day of OlPC, parasite burden obtained was 1.09×10^5 p/g of intestines (not significant, $P > 0.05$), whereas in mice treated with 30 mg/kg/day and 40 mg/kg/day, parasite burden was 4.66×10^3 p/g of intestines (99.8% parasite burden reduction, $P < 0.05$) and 692 p/g of intestines (99.97% parasite burden reduction, $P < 0.01$) respectively (**Figure 4B**). Additionally, by Day 30 there was an overall reduction in parasite burden in each group compared to their burden at Day 10.

To cross-validate results obtained by qPCR and to confirm the presence of *C. parvum* oocysts in the intestines, flow cytometry was used to quantify the number of oocysts per gram of intestines (oo/g of intestines). Flow cytometry data analysis allowed to identify oocysts by their size and morphology (FSC-A and SSC-A) and to count them for each intestinal sample. In general, total parasite burdens calculated by flow cytometry were lower than those obtained by qPCR suggesting the presence of other stages of the parasite in the intestines that flow cytometry cannot distinguish. However, the results were supportive of those of the qPCR. Mice treated with PBS, 100 mg/kg paromomycin and 10 mg/kg miltefosine showed an oocyst burden of 1.46×10^6 oo/g of intestines, 2.3×10^5 oo/g of intestines and 4.53×10^5 oo/g of intestines respectively (**Figure 4A**).

In mice treated with 20 mg/kg/day of OlPC, 9.4×10^4 oo/g of intestines was detected which consisted in a 93.5% decrease in oocyst burden compared to the PBS treated group ($P < 0.01$). The reductions in oocyst burden increase in mice treated with 30 mg/kg/day or 40 mg/kg/day of OlPC were only 180 oo/g of intestines (99.98% reduction, $P < 0.01$) and 167 oo/g of intestines (99.98% reduction, $P < 0.01$) respectively (**Figure 4A**). In both cases an average 4-log reduction in oocyst burden was observed when compared to PBS treated mice. In surviving mice treated with 10 mg/kg miltefosine, oocyst burden at Day 30 was 1.4×10^5 oo/g of intestines which is comparable to their oocyst burden at Day 10 (**Figures 4A and 4B**). At Day 30, at 20 mg/kg/day of OlPC, oocyst levels were slightly lower (6.63×10^4 oo/g of intestines) than mice treated with 10 mg/kg/day of miltefosine, but this difference remained statistically non-significant (52.6% oocyst burden reduction, Fig. 4B). At 30 mg/kg/day of OlPC, oocyst burden (2.81×10^3 oo/g of intestines) increased compared to the oocyst burden at Day 10 but still represented a 98% oocyst burden reduction compared to the oocyst burden at Day 30 for 10 mg/kg miltefosine-treated mice (Fig. 4B). Finally, at 40 mg/kg/day of OlPC, mice showed the lowest oocyst burden in their intestines with 34 oo/g of intestine representing a 99.98% oocyst burden reduction when compared to the 10 mg/kg miltefosine-treated mice ($P < 0.01$, **Figure 4B**).

OlPC eliminates the presence of oocysts in histological sections of the ileum of C57BL/6 IFNγR-KO mice infected with C. parvum

To visually validate the presence of *C. parvum* life cycle stages in the intestines of infected mice, the ileum was collected from all mice at time of death. Sections from the ileum were prepared and stained (H&E) for histopathology purposes. Ileum sections from mice treated with PBS and 100 mg/kg/day of paromomycin at Day 10 revealed an overwhelming presence of *C. parvum* life cycle stages and severe damage to the epithelium of the intestine (**Figures 5A and 5B**). Damage to intestinal mucosa includes blunting of the villi, acute inflammation and formation of fibrin (fibrinous hemorrhagic enteritis) resulting from local hemorrhage and clotting (**Figures 5A and 5B**). The ileum of mice treated with 10 mg/kg/day of miltefosine was as severely damaged and infected as the other controls (**Figure 5C**).

At Day 10, *C. parvum* oocysts were also abundantly present in mice treated with 20 mg/kg/day of OlPC and intestinal damage was also present but to a lesser extent than in the infected controls (**Figure 6A**). At the dose of 30 mg/kg/day of OlPC, mild intestinal damage and inflammation were noticeable and *C. parvum* oocysts were scarcely visible (**Figure 6B**). Finally, at 40 mg/kg/day, mice displayed practically no signs of intestinal damage or inflammation and a rare presence of *C. parvum* oocysts at Day 10 (**Figure 6C**). Slides from the ileum of non-infected controls were also prepared to provide a negative reference for comparison.

At Day 30, the surviving mice treated with 10 mg/kg/day of miltefosine revealed an abundant presence of *C. parvum* oocysts and severe damage to the gut epithelium (**Figure 7A**). At the lowest concentration of OlPC, mice displayed a moderate presence of *C. parvum* oocysts and only mild damage to the intestinal epithelium (**Figure 7B**). At 30 and 40 mg/kg/day of OlPC, mice showed a complete absence of *C. parvum* oocysts and a healthy ileum in comparison with controls (**Figures 8A and 8B**). Sections of ileum, liver and spleen were also processed in uninfected (control) mice treated with 40 mg/kg/day for 10 days and no inflammation or toxicity was noticeable.

Residual infectious oocyst concentration from mice exposed to OlPC is not sufficient to cause a new infection in naïve C57BL/6 IFNγR-KO mice

Another experiment was conducted to determine whether there was a sufficient number of infectious oocysts (more than the minimal infectious dose) remaining in the intestines of surviving mice at Day 10 or Day 30 to transmit the parasite and cause a new infection. To do so, naïve C57BL/6 IFNγR-KO mice were infected with a constant volume of the purified oocysts from mouse intestinal samples coming from the different treatment groups from both Day 10 and 30. In order to determine if naïve mice were infected, stool samples were taken once a week, processed and analyzed by qPCR and light microscopy (**Table 1**).

Table 1: *Infectivity of purified intestinal samples from the in vivo study in naïve C57BL/6 IFNγR-KO mice.*[a]*Treatment given to mice during the in vivo study. Purified intestinal samples from these mice at Day 10 or Day 30 were used to infect naïve C57BL/6 IFNγR-KO mice.* [b]*Represents the day post-inoculation that the stool from naïve mice was collected. The presence of C. parvum oocysts or its DNA in the stool of naïve mice is scored qualitatively. Dashes represent no available data due to mouse mortality during study: either there was no purified intestinal sample available to inoculate naïve mice (i.e.: no PBS-treated mice in the in vivo study survived until Day 30) or naïve mice died during rechallenge study (no naïve mice inoculated with purified intestinal samples from mice treated with miltefosine survived until stool collection on Day 35).*

		Day 10[b]		Day 17[b]		Day 35[b]	
Treatment[a]		Microscopy	qPCR	Microscopy	qPCR	Microscopy	qPCR
PBS	Day 10	Yes	Yes	-	-	-	-
	Day 30	-	-	-	-	-	-
100 mg/kg of paromomycin	Day 10	Yes	Yes	-	-	-	-
	Day 30	-	-	-	-	-	-
10 mg/kg of miltefosine	Day 10	Yes	Yes	-	-	-	-
	Day 30	No	No	Yes	Yes	-	-
20 mg/kg of OlPC	Day 10	Yes	Yes	-	-	-	-
	Day 30	No	No	Yes	Yes	-	-
30 mg/kg of OlPC	Day 10	Yes	Yes	-	-	-	-
	Day 30	No	No	Yes	Yes	-	-
40 mg/kg of OlPC	Day 10	No	No	No	Yes	-	-
	Day 30	No	No	No	No	No	No

Presence of *C. parvum* oocysts or DNA in the stool of these naïve mice suggests that a fully complete life cycle was achieved by the parasite. Therefore, the concentration of infectious oocysts in the purified intestinal samples of mice from the *in vivo* study would be considered to cause a new infection. Results demonstrated that only the purified intestinal samples from 40 mg/kg/day OlPC-treated mice at Day 30 were not able to successfully transmit a *C. parvum* infection in naïve C57BL/6 IFNγR-KO mice. For these inoculated naïve mice, no oocysts could be seen by light microscopy and no DNA could be detected by qPCR in the stool even by Day 35 (**Table 1**). Additionally, no sign of illness were observed and the histological sections of the ileum revealed a complete absence of oocysts.

This observation corroborates previous results as 40 mg/kg/day OlPC-treated mice have been shown to have practically cleared the infection at D30. However, naïve C57BL/6 IFNγR-KO mice infected with purified intestinal samples from other treatment groups developed signs of cryptosporidiosis. By Day 17, these inoculated naïve mice exhibited signs of illness (hunched back, weight loss, dehydration, lethargy and watery stools). Moreover, oocysts could be seen by light microscopy and detected by qPCR (**Table 1**). Histological sections revealed a heavy presence of *C. parvum* oocysts and severe damage to the microvilli. These results suggested that the oocysts in the purified intestinal samples of the other groups were sufficient to transmit *C. parvum* to a new host and cause acute cryptosporidiosis.

Discussion

C. parvum is a recognized threat to public health and has been reported in more than 40 countries in all five continents. Many waterborne outbreaks reported in the past decade do not limit *C. parvum* infection to developing countries, but have been reported in Canada, United States of America, Sweden and France as a result of tap water contamination. On one hand, *Cryptosporidium* causes persistent diarrhea and stunting in young children and, on the other hand, AIDS patients can develop a chronic infection that can be fatal. The major issue comes from the lack of efficacious treatment options for cryptosporidial infections.

The present example addressed the need for a new efficacious treatment against cryptosporidiosis by testing the compound OlPC, an alkylphosphocholine drug, already in clinical development against leishmaniasis. As a close analog of miltefosine, a drug recently approved by the FDA to treat cutaneous, mucosal and visceral leishmaniasis (2014), OlPC is suspected to share a similar mode of action by interfering with parasitic lipid biosynthesis and cellular membrane integrity while inducing apoptosis of the parasite. Repurposing OlPC as an anti-cryptosporidiosis drug would have several advantages as the safety of this compound is already reported and pharmacological as well as pharmacokinetics studies are already ongoing (Fortin et al., 2012).

Initial *in vitro* results demonstrated that OlPC efficiently inhibits *C. parvum* infection of HCT-8 cells after 48h exposure to the drug. OlPC reduced parasite burden by 84% at $\geq$ 10 µM and exhibited significant activity down to 10 nM with an EC_{50} of 18.84nM. The concentrations used for OlPC and miltefosine to achieve a significant reduction in *C. parvum* infectivity were far lower than the one published in the literature for paromomycin (EC_{50} of 711 µM), the currently used drug against cryptosporidiosis in AIDS patients. The toxicity profile displayed by OlPC was also very low with a 40% reduction in host cell viability only at 100 µM and higher. Conversely, it was reported that HCT-8 cells presented signs of toxicity when exposed

to miltefosine for 48h at concentrations as low as 24.5µM. Therefore, OlPC surpasses miltefosine in regards to its safety in host cells.

Even though many drugs have been shown to successfully inhibit *C. parvum* infection *in vitro*, many of them have failed to inhibit cryptosporidiosis in an animal model. Such is the case for monensin and paromomycin which only started to show efficacy in dexamethasone immunosuppressed mice at very high doses of 1g/kg/day. Because the action of the drugs on *C. parvum* parasites is more direct when tested *in vitro* than in an animal model, it is possible that the results from the former do not translate to the latter. In fact, many factors influence the effect of drugs *in vivo* on pathogens such as its bioavailability, pharmacodynamics and pharmacokinetics as well as food and drug interactions. Thus, it is important to understand the limitations of the *C. parvum in vitro* model to mimic an *in vivo* model of infection and we must acknowledge *in vitro* results only as informative precursors to further investigations in animal models.

Therefore, to determine whether the *in vitro* activity of OlPC on *C. parvum* could be translated *in vivo*, it was decided to use C57BL/6 IFNγR-KO mice, an animal model highly susceptible to *C. parvum* infection. Unlike other immunocompromised animal models where a very large *C. parvum* inoculum must be given (such as the dexamethasone, the SCID, the neonatal or the malnourished models), C57BL/6 IFNγR-KO mice can consistently develop severe illness with an infectious dose of only 10 oocysts. It has also been demonstrated that doses as low as 1000 oocysts were lethal by Day 9 to14 and 1500 oocysts lead to 80% death in these mice.

In the present example, mice received 4000 oocysts by oral gavage and began receiving treatment at Day 3 for a period of 10 consecutive days. By increasing the inoculum in these mice, 100% mortality was achieved in PBS treated mice by Day 10 which allowed to clearly identify the efficacy of the treatment. As early as Day 10, major differences between mice treated with the highest doses of OlPC (30 mg/kg/day and 40 mg/kg/day) with other groups could already be seen. Not only did 40 mg/kg/day of OlPC reduce parasite and oocyst burden in the intestinal tract by 99.6% and 99.98% respectively after only seven days of daily treatment (Day 10), it also allowed 100% of these mice to survive until the experimental endpoint (Day 30) ($P < 0.001$). At Day 30, parasite and oocyst burden in the intestines of the 40 mg/kg/day OlPC-treated group reached a 99.97% and 99.98% reduction respectively ($P < 0.001$) in comparison with the 10 mg/kg miltefosine group.

These data firmly support that, even after the completion of the treatment with OlPC, mice did not show any sign of relapse in parasite burden or recurrence in clinical symptoms. In addition, a complete elimination of oocyst shedding in the stools was observed and no parasite was noticed by light microscopy of histology section of ileum of 40 mg/kg/day OlPC-treated mice at Day 30. In consequence, it is not surprising that purified intestinal samples from 40 mg/kg/day OlPC-treated mice at D30 were incapable of further transmitting the infection to naïve C57BL/6

IFNγR-KO mice. Together, this validates the hypothesis that OlPC rescued/cured immunocompromised mice from a lethal infection with *C. parvum* and, even if the 40 mg/kg/day OlPC treatment did not completely cleared infection at Day 30 (**Figure 4B**), it is not likely that the surviving parasites can cause a recrudescence of infection if mice were not sacrificed at Day 30.

In short, a stringent animal model was used that clearly discriminated if a drug was sufficiently potent to rescue mice from a lethal infection. In this model, paromomycin, given at 100 mg/kg/day for 10 days, failed to rescue mice, whereas in a neonatal model, where the same dosage of paromomycin was given for 6 days, it was able to reduce oocyst burden in the distal colon by 97% and oocyst shedding by 96%. However, these numbers are not corroborated by clinical cases, where paromomycin treatments are associated with a high probability of relapse in AIDS patients.

At lower doses, 30 mg/kg/day and 20 mg/kg/day of OlPC, treatments were still able to keep 75% of infected mice alive at Day 30, but were not able to eliminate oocyst shedding. Moreover, purified intestinal samples from these mice were capable of transmitting the infection to other naïve C57BL/6 IFNγR-KO mice. This suggests that the dose of these OlPC treatment regimens were not sufficient to eliminate *C. parvum* parasites or to prevent a recurrent infection after the end of the treatment (even though there was a 98% oocyst burden reduction in the intestines by Day 30).

Finally, miltefosine was also tested at 10 mg/kg/day for 10 days as a control because of the similar properties and structure it shares with OlPC. Results demonstrated that it had modest, but significant level of activity against *C. parvum* infection since it was able to rescue 50% of infected mice at Day 30. However, the surviving mice were still heavily infected and presented severe signs of illness.

No sign of discomfort or behavioral changes were noted in infected and uninfected mice treated with 40 mg/kg/day of OlPC for 10 days, which is supportive of data previously obtained in a mouse model of leishmaniasis. Additionally, sections of the ileum, liver and spleen from these mice showed no signs of toxicity or inflammation associated to OlPC (data not shown).

The present example did not compare miltefosine and OlPC at the same dosages across the entire range. While this may be perceived as a limitation, it was reflective of concerns regarding miltefosine toxicity at higher doses.

Conclusion

The strong activity of OlPC on *C. parvum* parasites *in vitro* was thoroughly supported by the outcome of the animals in *in vivo* study as OlPC rescues C57BL/6 IFNγR-KO mice from a lethal infection of *C. parvum.* Data obtained by qPCR from the *in vivo* study was not only cross-validated by flow cytometry and light microscopy, but was also confirmed visually by

histological sections from mice ileums. Furthermore, OlPC demonstrated, at its highest dose, a lasting effect and mice showed no sign of relapse of infection even 17 days after the end of the treatment. Together, this establishes OlPC as novel, consistent, sustainable, safe, and potent anti-cryptosporidial treatment option for immunocompromised patients.

5

CLAIMS

1. Oleylphosphocholine (OlPC) for use in the prevention or treatment of cryptosporidiosis in a vertebrate.

2. Oleylphosphocholine (OlPC) for use according to claim 1 wherein the causative agent of said cryptosporidiosis is *Cryptosporidium parvum*.

3. Oleylphosphocholine (OlPC) for use according to claim 1 or claim 2, wherein said vertebrate is an immunocompromised vertebrate.

4. Oleylphosphocholine (OlPC) for use according to any of the claims 1 to 3, wherein said vertebrate is a human.

5. Oleylphosphocholine (OlPC) for use according to any of the claims 1 to 4, wherein said prevention or treatment comprises oral administration of oleylphosphocholine (OlPC).

6. Method for preventing or treating cryptosporidiosis in a vertebrate comprising administering a therapeutically effective dose of oleylphosphocholine (OlPC) to said vertebrate.

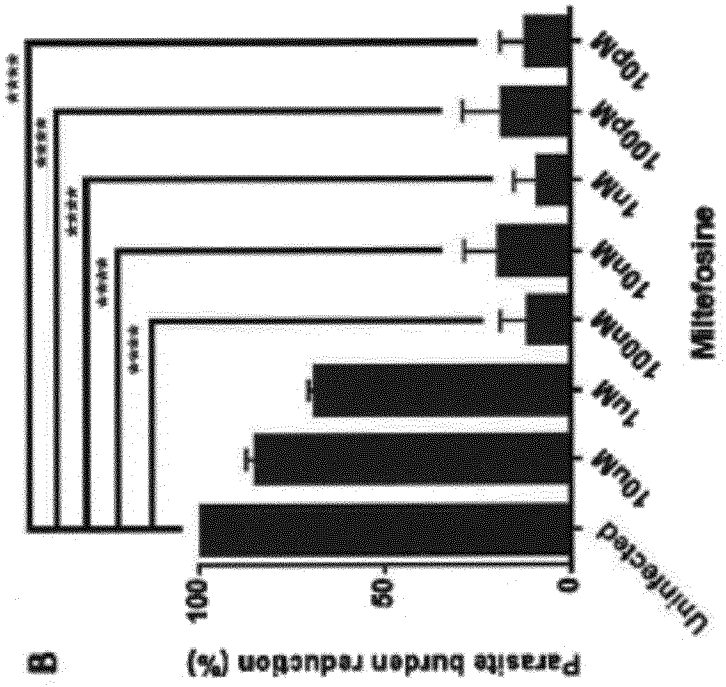
7. Method according to claim 6, wherein said vertebrate is an immunocompromised vertebrate.

8. Method according to claim 6 or claim 7, wherein said vertebrate is a human.

9. Method according to any of the claims 6 to 8 comprising oral administration of said oleylphosphocholine (OlPC).

10. Method according to any of the claims 6 to 9, wherein the causative agent of said cryptosporidiosis is *Cryptosporidium parvum*.

FIGURE 1


B
Parasite burden reduction (%)
100
50
0
Uninfected
10uM
1uM
100nM
10nM
1nM
100pM
10pM
Miltefosine
****

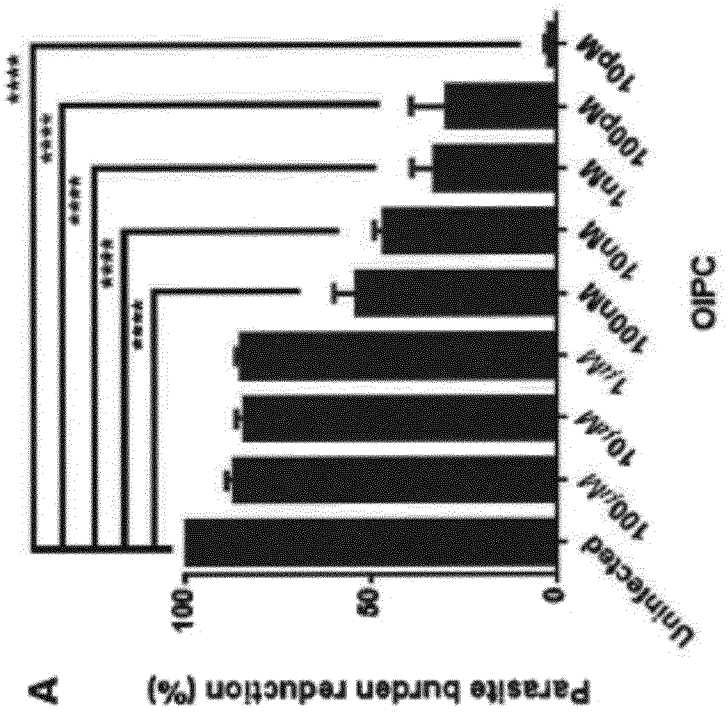

A
Parasite burden reduction (%)
100
50
0
Uninfected
100uM
10uM
1uM
100nM
10nM
1nM
100pM
10pM
OIPC
****

FIGURE 2

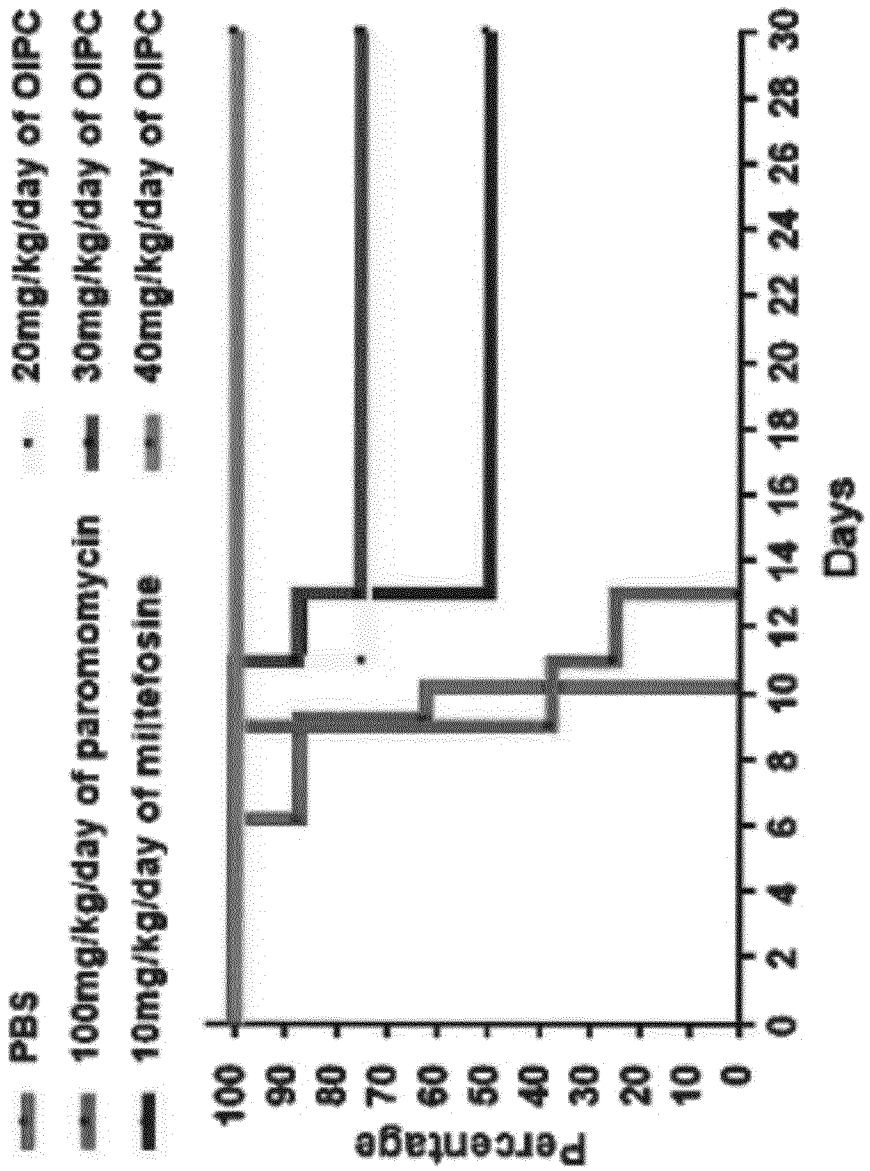

PBS
20mg/kg/day of OIPC
100mg/kg/day of paromomycin
30mg/kg/day of OIPC
10mg/kg/day of miltefosine
40mg/kg/day of OIPC
Percentage
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days

FIGURE 3

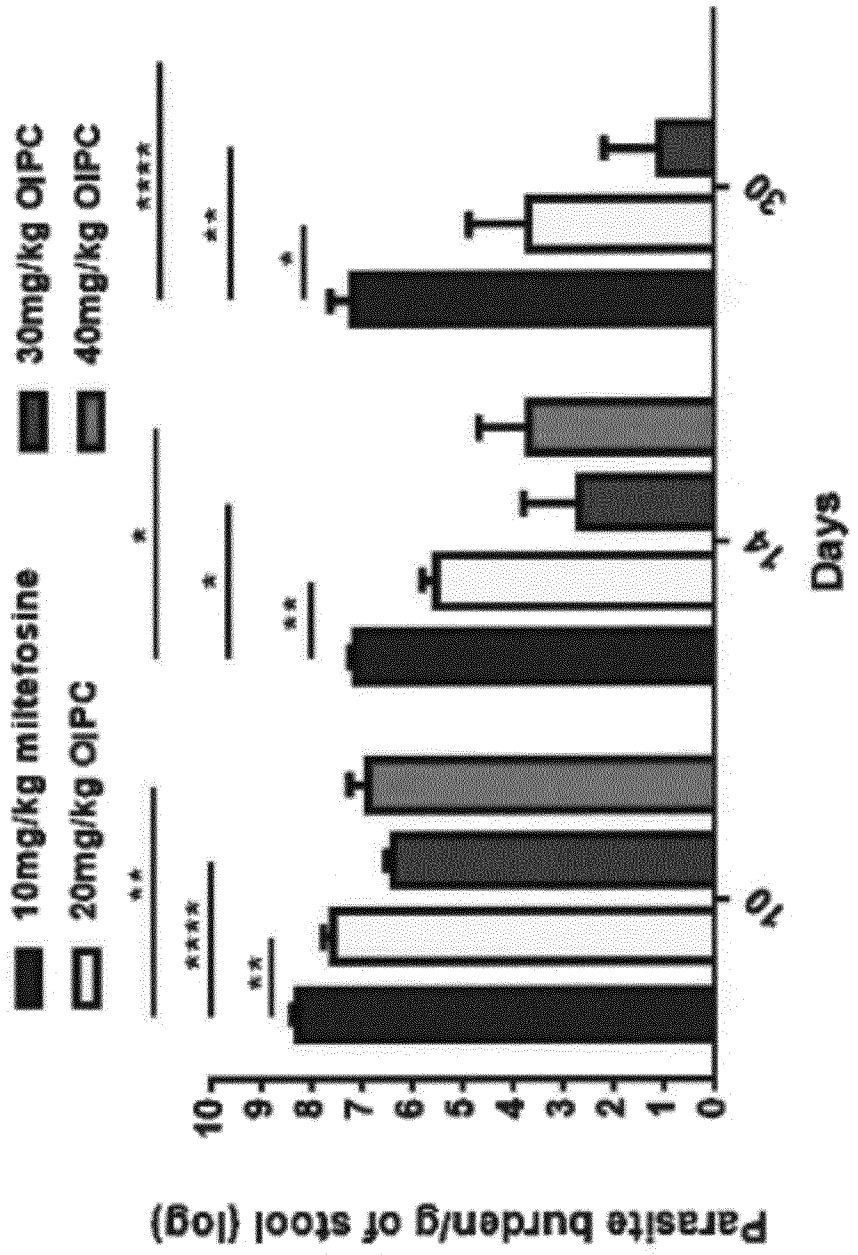

10mg/kg miltefosine
20mg/kg OlPC
30mg/kg OlPC
40mg/kg OlPC
Parasite burden/g of stool (log)
Days
10
14
30

FIGURE 4

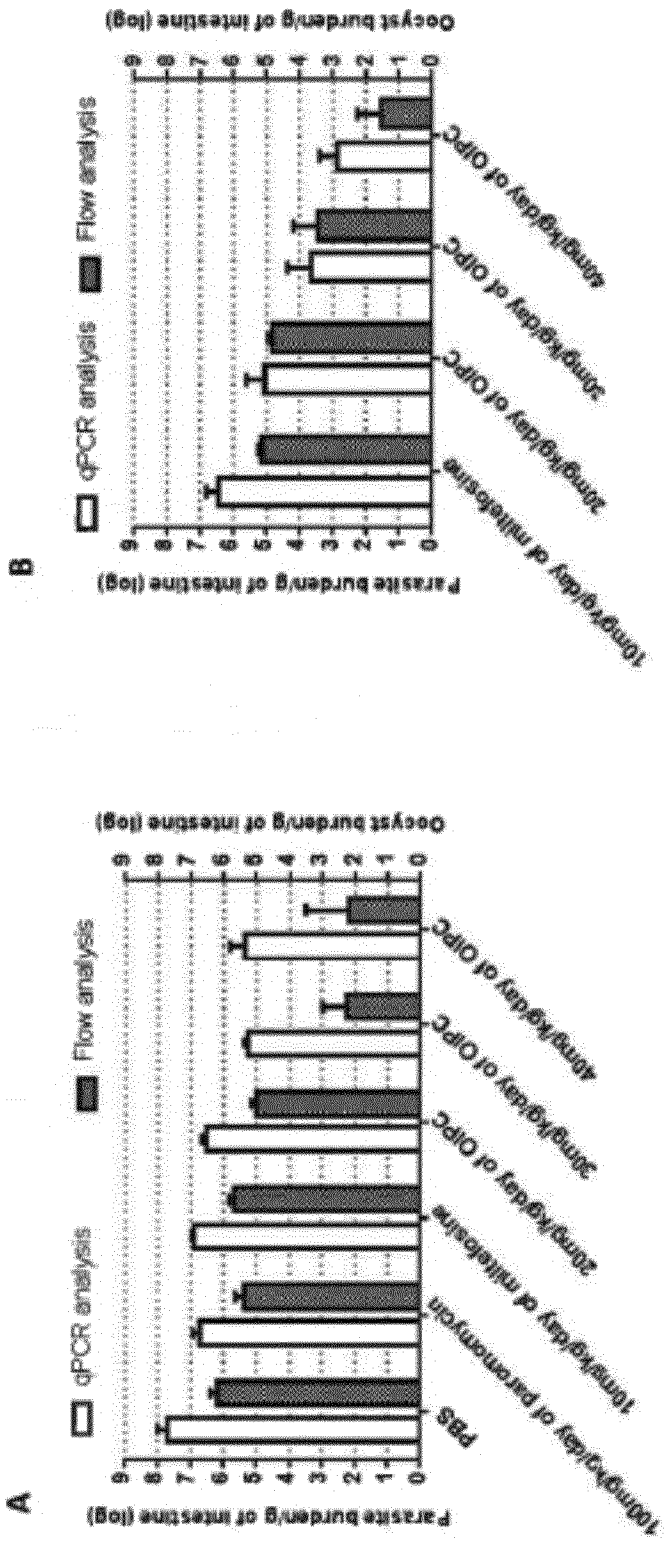

A
B
qPCR analysis
Flow analysis
Parasite burden/g of intestine (log)
Oocyst burden/g of intestine (log)
PBS
100mg/kg/day of paromomycin
10mg/kg/day of nitazoxanide
20mg/kg/day of nitazoxanide
30mg/kg/day of OlPC
40mg/kg/day of OlPC

FIGURE 5

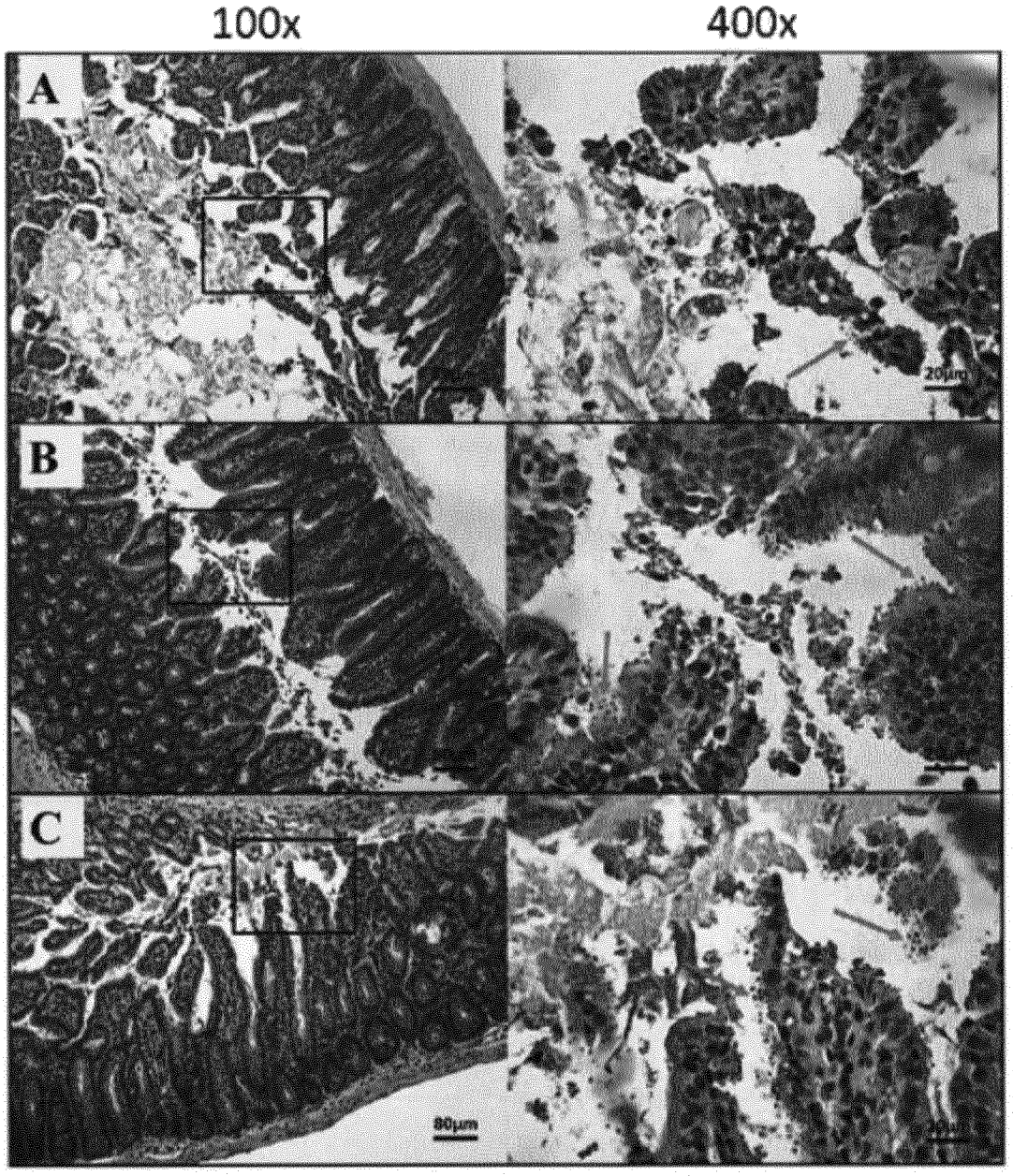


FIGURE 6

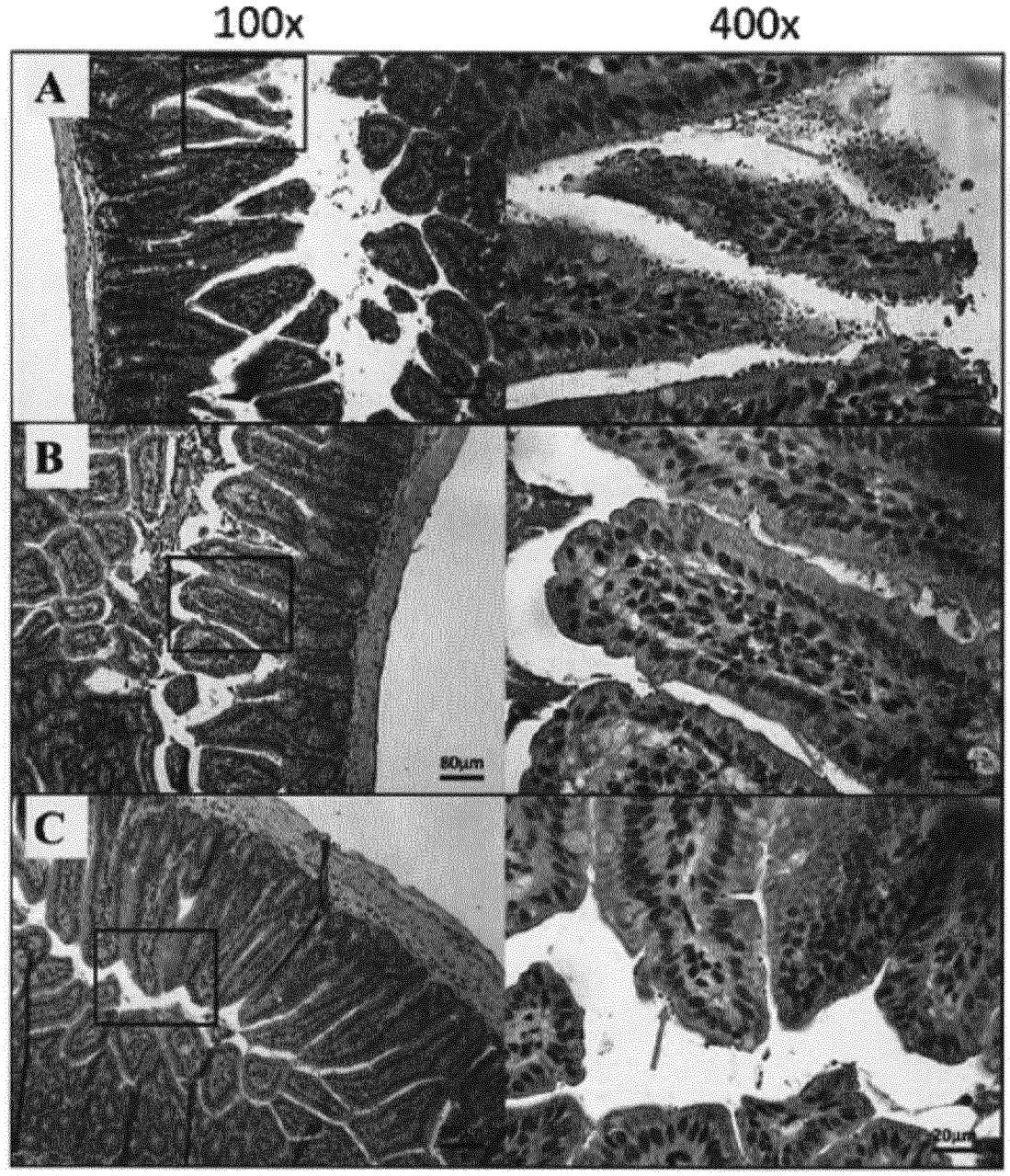


FIGURE 7

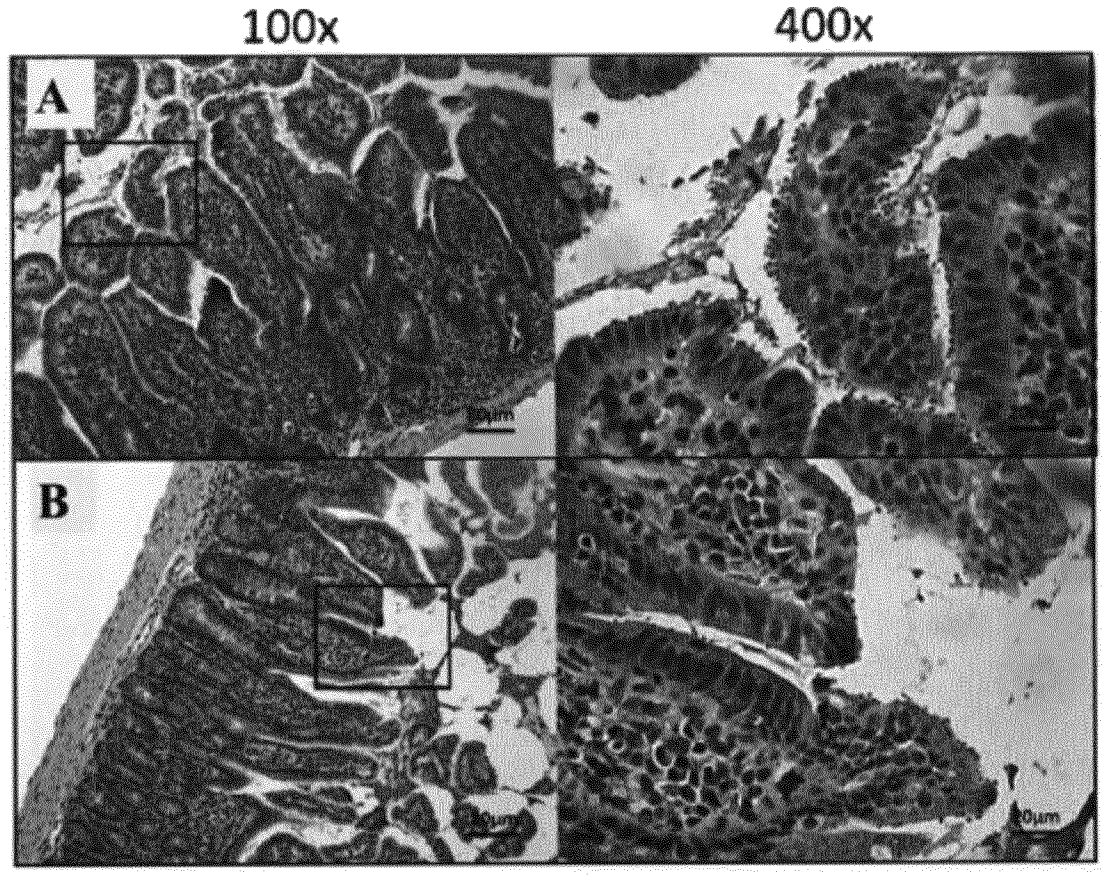


FIGURE 8