Supplemental material

Materials and Methods

Host cells, parasite and reagents

In vitro culture of *C. parvum* was performed as described (1-3). Briefly, HCT-8 cells (ATCC CCL-244) and Caco-2 cells (ATCC HTB-37) were used to host the growth of parasite *in vitro*. Host cells were maintained in T-75 tissue culture flasks with RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C under 5% CO_2 atmosphere. The *C. parvum* oocysts (BGF-1 strain; subtype IIaA17G2R1) were purchased from Bunch Grass Farm (Deary, ID, USA). Oocysts were further purified with 10% Clorox on ice for 8 min and washed 5-8 times with dH_2O by centrifugation and stored at 4°C. Oocysts less than 3 months old since harvest were used in experiments. Occidiofungin was purified from liquid culture of *B. contaminans* MS14 and lyophilized for long-term storage as described elsewhere (4). Stock solution of occidiofungin (10 mM) was prepared in dimethyl sulfoxide (DMSO) before use. All chemicals used in this study were purchased from Sigma-Aldrich or as specified.

In vitro drug efficacy assay against *C. parvum*

In vitro drug efficacy was determined by a 44-h infection assay coupled with qRT-PCR to determine parasite loads as described (1, 2). Briefly, host cells were seeded into 96-well plates (25,000 cells/well), incubated at 37°C overnight to reach 80-90% confluence (~40,000 cell/well). Purified *C. parvum* oocysts were added into plates at 20,000 oocysts/well (host cell to oocyst ratio at ~2), and allowed for excystation and invasion for 3 h. At this time point, uninfected parasites were removed by a medium change, and occidiofungin was added to well at specified final concentrations. Cells were incubated at 37°C for additional 41 h (= total 44 h infection time), followed by preparation of cell lysates with 150 µL of iScript qRT-PCR sample preparation reagent (iScript lysis buffer) (Bio-Rad). Paromomycin (100 µg/mL) was used as a positive control, and all wells contained 0.5% DMSO diluent.

Effect of pre-treatment of host cells with Occidiofungin on parasite growth

HCT-8 cells in 96-well plates at 80-90% confluence (~40,000 cells/well) were incubated with 200 nM occidiofungin in culture medium for 24 h, respectively. At the end of each treatment,

cells were washed with three times with PBS to remove drug residual and inoculated with *C. parvum* oocysts (50,000 oocysts/well). Cells were washed with PBS and lysed at in 100 µL iScript lysis buffer at 3, 9 and 18 hpi, respectively.

Effect of occidiofungin on various developmental stages of *C. parvum*

In vitro culture of *C. parvum* was performed in 96-well plates as described above and treated with 200 nM of occidiofungin for 0-3, 0-6 and 0-9 hour durations post-infection (**hpi**). For each hpi treatment group, HCT-8 cells were infected with 100,000 oocysts/well, and 200 nM occidiofungin was added to the infection medium together with *C. parvum* oocysts, and cells were then lysed in 150-µL of iScript lysis buffer at the end of each treatment. For the 3-22 hpi and 3-44 hpi treatment groups, host cells were infected with 50,000 oocysts/well, followed by medium change with 200 nM compound at 3 hpi. Cells were lysed in iScript lysis buffer at 22 and 44 hpi, respectively. For 18-44 hpi treatment group, compound was added at 18 hpi and cells were lysed at 44 hpi.`

In vitro drug withdrawal assay

HCT-8 cells (~40,000 cells/well) were infected with *C. parvum* at 20,000 oocysts/well. Occidiofungin (200 nM) was added at 3 hpi and removed by a medium exchange at 6, 9, 12 and 18 hpi, respectively. In all groups, parasites were allowed to grow up to 44 hpi. The group receiving a full course of treatment (3-44 hpi) was included as control. For all treatment groups, cells were lysed at 44 hpi in 150 µl iScript lysis buffer.

Effect of occidiofungin on *C. parvum* sporozoites and invasion of sporozoites

Free sporozoites were prepared by incubating oocysts in PBS containing 0.25% trypsin and 0.5% taurodeoxycholic acid at 37°C for 1 h, followed by washes with culture medium as described (2). Sporozoites were treated with 200 or 400 nM occidiofungin at 37°C for 40 min in culture medium, followed by washes with culture medium for three times. Host cells were infected with treated sporozoites (sporozoite/cell ratio = 4:1) for 2 or 6 hpi. Cells were washed with PBS and then lysed in 40 µl iScript lysis buffer. Sporozoites treated with 0.5% DMSO were used as negative control. The morphology of sporozoites were also examined by differential interference and indirect fluorescence microscopy using a rabbit polyclonal antibody against total sporozoite

membrane proteins and a goat anti-rabbit IgG secondary antibody conjugated with tetramethylrhodamine isothiocyanate (TRITC) as described (5, 6).

In vitro cytotoxicity of occidiofungin on HCT-8 cells

The cytotoxicity of occidiofungin on HCT-8 was evaluated using an MTS assay as described.(7) Briefly, host cells were cultured in 96-well plates overnight until ~80-90% confluence and treated with serially diluted occidiofungin for 44 h. After three washes with PBS, cell viability was monitored using the Cell Titer 96 AQ_{ueous} one solution cell proliferation kit according to the manufacturer's instructions (Promega, Madison, WI).

Determination of parasite loads by qRT-PCR

The parasite loads were determined by qRT-PCR detection of parasite 18S (Cp18S) rRNA from diluted cell lysates using reagents and procedures as described (1, 2). Host cell 18S (Hs18S) rRNA was also detected for normalization. Real-time qRT-PCR reactions were performed in a Bio-Rad CFX384 Touch Real Time PCR Detection System. The relative parasite loads were determined based on the $\mathbf{\Delta\Delta C_T}$ values between Cp18S and Hs18S transcripts and between experimental and control groups as described (1, 2).

Oral stability study

Occidiofungin was formulated using 5% 2-hydroxypropyl-β-cyclodextrin in 0.3% noble agar. Occidiofungin was administered by oral gavage at a 50 mg/kg/day dose to BALB/c mice for three days (3 males and 3 females). The classic antifungal fuconazole at 50 mg/kg was used as a control. Another six mice were used as vehicle control. Daily fecal sample collection started at 10 h post administration on the third day for three days. An Agilent 1200 front-end chromatography system and a TSQ Quantum Access triple quadrupole mass spectrometer was used to analyze and determine occidiofungin concentration in fecal samples. The weight of each mouse was measured daily at the time of fecal collection. Mice were monitored daily for behaviors indicating pain and suffering (nesting, grooming, or lethargy). The mouse studies were approved by the Institutional Animal Care and Use Committees of Texas A&M University (IACUC 2018-0319).

Statistical analysis

At least three biological replicates, each with two technical replicates for qRT-PCR, were included in all experiments. Each assay was performed for at least three times independently. Data were analyzed using GraphPad Prism (version 5.0f or higher). Statistical significances were assessed by two-way ANOVA and Sidak's multiple comparison test.

References

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