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EXPERIMENTAL THERAPEUTICS

Novel Antiparasitic Activity of the Antifungal Lead Occidiofungin

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ABSTRACT Novel antiparasitic activity was observed for the antifungal occidiofungin. It efficaciously and irreversibly inhibited the zoonotic enteric parasite *Cryptosporidium parvum in vitro* with limited cytotoxicity (50% effective concentration [EC_{50}] = 120 nM versus 50% cytotoxic concentration [TC_{50}] = 988 nM), and its application disrupted the parasite morphology. This study expands the spectrum of activity of a glycolipopeptide named occidiofungin. Occidiofungin has poor gastrointestinal tract absorption properties, supporting future investigations into its potential activities on other enteric parasites.

KEYWORDS protozoan parasite, *Cryptosporidium parvum*, occidiofungin, efficacy, cytotoxicity, *in vitro*

The glycolipopeptide occidiofungin was originally isolated from *Burkholderia contaminans* for antifungal activity (Fig. 1A) (1). It has a broad spectrum of activity against fungal pathogens, including species in the genera *Alternaria*, *Aspergillus*, *Fusarium*, *Geotrichum*, *Macrophomina*, *Microsporum*, *Penicillium*, *Pythium*, *Rhizoctonia*, and *Trichophyton*, with MICs of 1 to 32 μg/ml (or MIC_{50}s of 0.25 to 16 μg/ml), and various *Candida* species (MICs at 0.5 to 2.0 μg/ml) (1–3).

Occidiofungin is poorly absorbed by the digestive system (4) but highly efficacious *in vivo* against vulvovaginal *Candida albicans* infection in mice when administered intravaginally (3). The mode of action of occidiofungin is distinct from those of the four common classes of antifungal agents (5). Occidiofungin disrupts fungal membrane morphology and induces apoptosis (1, 6). A recent study identified actin filaments as the primary cellular target of occidiofungin in fungi (3).

The broad-spectrum antifungal activity of occidiofungin and its retention in the gastrointestinal tract (GIT) prompted us to explore its potential therapeutic value against the enteric parasite *Cryptosporidium parvum*, a globally important zoonotic protozoan that has a unique epicellular parasitic lifestyle (7–9).

In this study, HCT-8 and Caco-2 cells were used to grow *C. parvum in vitro* in 96-well plates (10–12). *C. parvum* oocysts (subtype IIaA17G2R1) <3 months old were used in experiments. Occidiofungin was purified from *B. contaminans* (3). *In vitro* drug efficacy was evaluated by a 44-h infection assay coupled with quantitative reverse transcription-PCR (qRT-PCR) to determine parasite loads (10, 11). Paromomycin (140 μM) was used as a positive control. *In vitro* cytotoxicity for host cells was evaluated by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (13). The oral stability of occidiofungin was evaluated in BALB/c mice by oral gavage at a dosage of 50 mg/kg of body weight/day for 3 days (3 males and 3 females), together with vehicle control and positive control using fluconazole (50 mg/kg/day).

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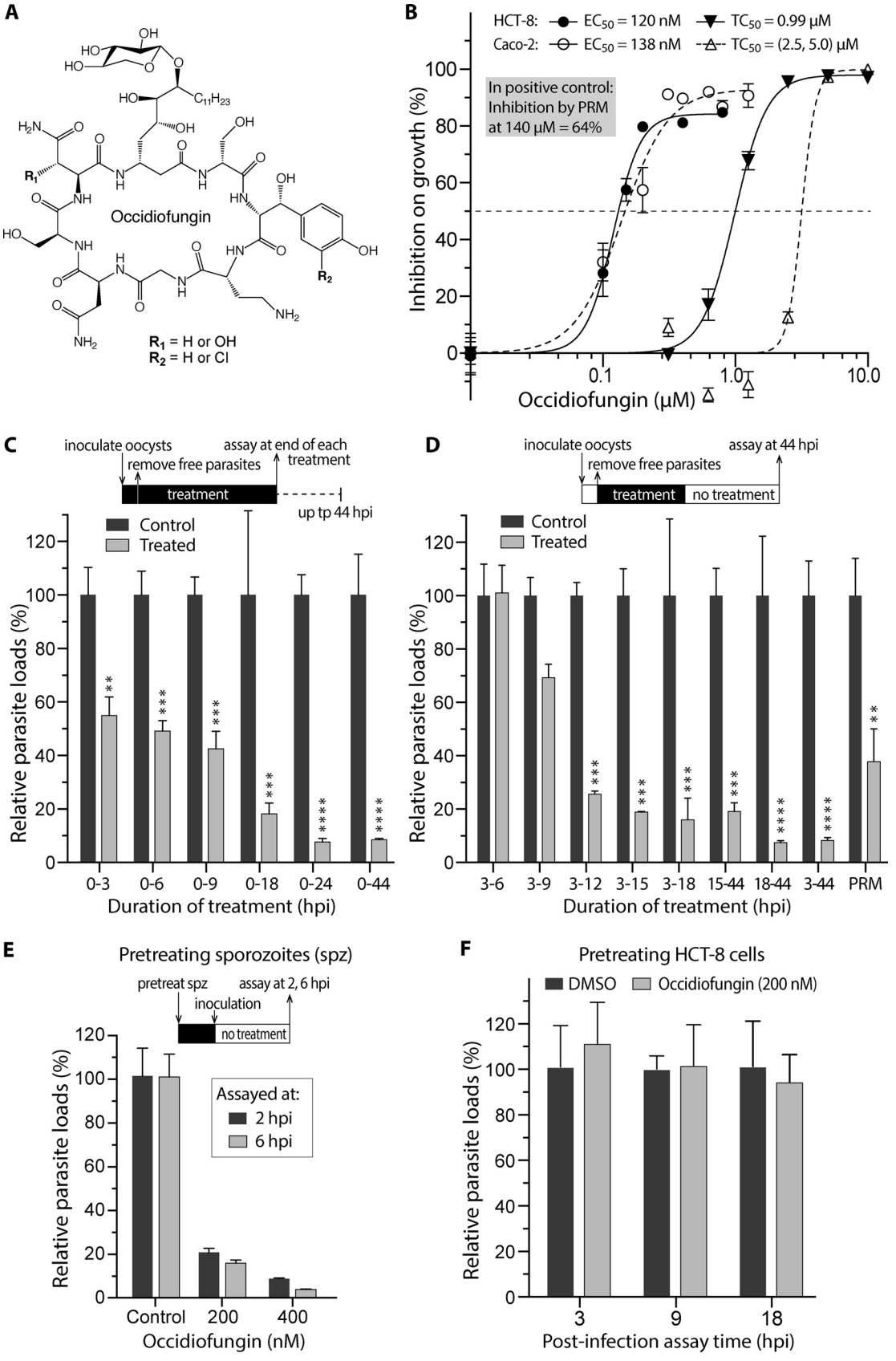

FIG 1 Activity of occidiofungin against the growth of *Cryptosporidium parvum in vitro*. (A) Covalent structure of occidiofungin, a glycolipopeptide. (B) *In vitro* efficacy of occidiofungin against *C. parvum* cultured in two host cell lines (HCT-8 and Caco-2) and

(Continued on next page)

Feces were collected 10 h postadministration on the third day for determining occidiofungin concentrations by chromatography-mass spectrometry. All *in vitro* assays included ≥3 biological replicates and were performed ≥3 times independently. Data were assessed by two-way analysis of variance (ANOVA) and Sidak's multiple-comparison test. A more detailed description of materials and methods is provided in Supplemental Text S1.

In a 44-h infection assay, occidiofungin displayed low-nanomolar anticryptosporidial activity (50% effective concentration [EC_{50}] = 120 and 138 nM for parasites cultured in HCT-8 and Caco-2 cells, respectively) (Fig. 1B), which was more potent than its antifungal efficacy (MIC_{50} = 0.25 to 16 μg/ml; equivalent to ~230 to 14,500 nM) (1, 2). The anti-*Cryptosporidium* potency was comparable to that of the most efficacious known lead compounds, which typically had EC_{50}s in the low-nanomolar to single-digit-micromolar range (e.g., phosphatidylinositol 4-kinase [PI4K], histone deacetylase [HDAC], and long-chain fatty acyl coenzyme A [ACS] inhibitors at 0.1 to 0.2 μM; calcium-dependent protein kinase [CDPK], lysyl-tRNA synthetase [KRS], and methionyl-tRNA synthetase [MetRS] inhibitors at 1.3 to 6.0 μM) (13–19). Within the effective concentrations, occidiofungin was nontoxic to host cells, and it started to affect host cells only at micromolar levels (50% cytotoxic concentration [TC_{50}] = 0.99 μM on HCT-8 cells and 2.5 to 5.0 μM on Caco-2 cells) (Fig. 1B), giving safety intervals (calculated as TC_{50}/EC_{50}) of 8.3 to 18.

Occidiofungin acted on *C. parvum* in various merogonic developmental stages (Fig. 1C to E). When present during excystation and invasion, occidiofungin (200 nM) reduced the parasite loads by 45.1% in the 0- to 3-h-postinfection (hpi) treatment group, and the anti-*Cryptosporidium* activity increased over the treatment time (Fig. 1C). Drug withdrawal experiments indicated that occidiofungin acted on *Cryptosporidium* irreversibly, as the parasite was unable to recover its growth after 9-h or longer treatments followed by removal of compound and continuous parasite growth for up to 44 hpi (Fig. 1D). In positive controls, a full course of treatment with paromomycin (140 μM) inhibited the parasite growth by 62.2% (Fig. 1D), which was comparable to previous reported values (10).

The strong inhibitory effect of occidiofungin in 0- to 3- and 0- to 6-hpi groups shown in Fig. 1C (versus no effect in the 3- to 6-hpi group [Fig. 1D]) suggested its action on sporozoites, a motile infectious stage of *C. parvum*. To test this hypothesis, we treated sporozoites with occidiofungin for 40 min at 37°C and removed the compound before examining their morphology and evaluating their invasion of host cells. We observed 79.2% (200 nM) and 84.1% (400 nM) reductions of parasite loads when they were assayed at 2 hpi and further reductions (91.2% and 94.1%) at 6 hpi (Fig. 1E).

Notably, occidiofungin also significantly deformed the sporozoite morphology (Fig. 2A). In the control group treated with 0.5% dimethyl sulfoxide (DMSO), sporozoites were rod shaped, whereas occidiofungin-treated sporozoites were significantly shortened and much more transparent, indicating the loss of cytosolic contents by disruption of cytoplasm membrane integrity. However, it remains to be determined whether occidiofungin might also act on the parasite actin, as observed in fungi (3).

The enteric *Cryptosporidium* parasites are distinguished by their epicellular devel-

FIG 1 Legend (Continued)
cytotoxicity. Paromomycin (PRM) at 140 μM was used as a positive control. (C) Effect of occidiofungin on the growth of *C. parvum in vitro* with various lengths of treatment. Compound was added to the medium along with oocyst inoculation for the specified durations of treatment, and the parasite loads were assayed at the end of each treatment. (D) Drug withdrawal assay. Occidiofungin was added at 3 hpi after the removal of uninvaded parasites. After treatment of infected cells for specified durations, compound was removed, and the parasites were allowed to grow and recover in the absence of compound for up to 44 hpi before the parasite loads were determined. PRM at 140 μM was used as a positive control. (E) Effect of pretreating sporozoites with occidiofungin on parasite infection. Free sporozoites were treated with occidiofungin at 200 and 400 nM for 40 min in culture medium and then allowed to invade host cells for 2 and 6 h. The parasite loads were determined at 2 and 6 hpi time points. (F) Effect of pretreating host cells with occidiofungin on parasite infection. HCT-8 cells were treated with occidiofungin at 200 nM for 24 h. After removal of compound, host cells were incubated with parasite oocysts and assayed at 3, 9, and 18 hpi. Parasite loads were determined by qRT-PCR. Statistical significance was evaluated by two-way ANOVA and Sidak's multiple-comparison test. Error bars represent standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

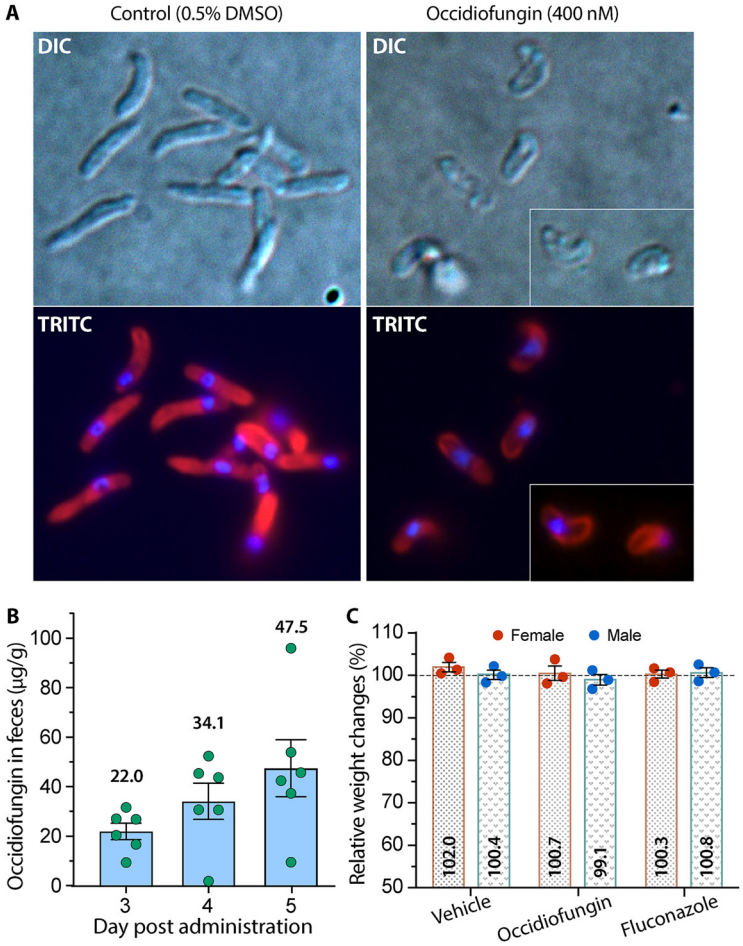

FIG 2 Action of occidiofungin on *Cryptosporidium parvum* sporozoites and oral stability of occidiofungin in mice. (A) Deformation of the morphology of sporozoites after treatment with occidiofungin at 400 nM for 40 min. All treatment groups contained 0.5% DMSO, including controls. DIC, differential interference contrast microscopy; TRITC, tetramethylrhodamine isothiocyanate (sporozoites were labeled by a rabbit polyclonal antibody against *C. parvum* total proteins and TRITC-conjugated secondary antibody). (B) Concentrations of occidiofungin in BALB/c mouse feces 10 h after a single oral daily dose (50 mg/kg) for 3 days. (C) Mouse weight gain or loss 10 h after the third dose of occidiofungin (versus fluconazole at 50 mg/kg/day).

opment in the GIT and the presence of extracellular stages. Therefore, the previously observed poor absorption of occidiofungin by the GIT could be advantageous in maximizing the activity of occidiofungin on the parasites (4). Indeed, the retention of occidiofungin in the mouse GIT was confirmed by an oral stability assay, in which feces collected at 10 h after oral administration of 50 mg/kg/day contained 22.0, 34.1, and 47.5 μg/g of occidiofungin following three consecutive daily doses (Fig. 2B). Pending further study on the relationship between drug absorption and retainment in the GIT, it was encouraging that the concentrations of occidiofungin in feces were theoretically ~15 to 33 times higher than the anticryptosporidial EC_{50}. Additionally, occidiofungin did not produce any signs of toxicity or significant weight loss in mice (Fig. 2C).

Cryptosporidiosis is a globally important zoonotic disease, and fully effective treatment is currently unavailable. There is an urgent need to develop new anticryptosporidial therapeutics. Occidiofungin has been pursued as a lead for developing antifungal therapeutics (e.g., candidiasis). The present study not only expands the activity spectrum of occidiofungin to include antiparasitic activity but also points out a potential new direction for developing anticryptosporidial therapeutics.

C. parvum is an epicellular enteric parasite separated from the host cell cytosol by an electron-dense band (7–9). It has been observed that some compounds in the

circulatory system might not effectively pass the electron-dense band to act on the parasite, and accumulation in the GIT is critical to anticryptosporidial activity for some compounds (e.g., paromomycin and bumped-kinase inhibitors) (20). Therefore, poor absorption is advantageous in that it allows occidiofungin to act more effectively on enteric *Cryptosporidium* or other parasites (4). It would also reduce potential organ-specific toxicities. Further, occidiofungin concentrations recovered from feces were 15 to 33 times higher than anticryptosporidial EC_{50}s, suggesting that oral administration of occidiofungin has potential for successfully treating *C. parvum* infections.

In summary, we report occidiofungin as a novel antiparasitic agent and a new lead for developing anticryptosporidial therapeutics. The anticryptosporidial activity of occidiofungin, together with its unique pharmacokinetic feature in the GIT, suggests that it could also be investigated for potential activity against other enteric parasites.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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L.S. is a board member of Sano Chemicals, Inc. Sano Chemicals is actively developing occidiofungin for the treatment of serious fungal infections.

G.Z., L.S., F.G., and H.Z. conceived the study design. J.M., F.G., H.Z., M.J., A.R., and R.O. conducted the experiments. J.M., G.Z., H.Z., and L.S. wrote the manuscript. All authors read and approved the manuscript.

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