Clinical Infectious Diseases

Clofazimine for treatment of cryptosporidiosis in HIV-infected adults (CRYPTOFAZ): an experimental medicine, randomized, double-blind, placebo-controlled phase 2a trial

--Manuscript Draft--

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Full Title:	Clofazimine for treatment of cryptosporidiosis in HIV-infected adults (CRYPTOFAZ): an experimental medicine, randomized, double-blind, placebo-controlled phase 2a trial
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Abstract:	Background: We evaluated efficacy, pharmacokinetics (PK), and safety of clofazimine (CFZ) in HIV-infected patients with cryptosporidiosis. Methods: We performed a randomized, double-blind, placebo-controlled study. Primary outcomes in Part A were reduction in Cryptosporidium shedding, safety, and PK. Primary analysis was according to protocol (ATP). Part B of the study compared CFZ PK in matched HIV-infected individuals without cryptosporidiosis. Results: Twenty Part A and 10 Part B participants completed the study ATP. Almost all Part A participants had high viral loads and low CD4 counts, consistent with failure of antiretroviral (ARV) therapy. At study entry, the Part A CFZ group had higher Cryptosporidium shedding, total stool weight, and more diarrheal episodes compared to the placebo group. Over the inpatient period, compared to those who received placebo, the CFZ group Cryptosporidium shedding increased by 2.17 log 2 Cryptosporidium per gram stool (95% upper confidence limit: 3.82), total stool weight decreased by 45.3 g (p=0.37), and number of diarrheal episodes increased by 2.32 (p=0.87). The most frequent solicited adverse effects were diarrhea, abdominal pain, and malaise. Three CFZ and 1 placebo subjects died during the study. Plasma levels of CFZ in participants with cryptosporidiosis were 2-fold lower than Part B controls. Conclusion: Our findings do not support the efficacy of CFZ for the treatment of cryptosporidiosis in a severely immunocompromised HIV population. However, this trial demonstrates a pathway to assess the therapeutic potential of drugs for cryptosporidiosis treatment. Screening persons with HIV for diarrhea, and especially Cryptosporidium infection, may identify those failing ARV therapy.
Response to Reviewers:	William A. Petri, MD Associate Editor, Clinical Infectious Diseases 9 March 2020 Dear Dr. Petri, Thank you for considering our original study titled: “Clofazimine for treatment of cryptosporidiosis in HIV-infected adults (CRYPTOFAZ): an experimental medicine, randomised, double-blind, placebo-controlled phase 2a trial” for publication in Clinical Infectious Diseases. We also thank the reviewers for their thoughtful comments, and believe the revised manuscript has been improved as a result. Below we have listed our response to reviewer comments: Reviewer #1: Drugs to treat Cryptosporidiosis in severely immunocompromised patients, particularly those with HIV/AIDS, are not available and are urgently needed. This study was an attempt to see whether an existing drug, Clofazamine (CFZ), which has given very promising results in animal models, would be effective in this population. There are clearly issues with this trial. Recruitment proved unexpectedly difficult, which resulted in smaller treatment and control groups than the authors wanted, although they reached the sample size (10 in each group) that they calculated would have sufficient power to give an 80% chance of detecting a significant difference between the groups. And, despite randomisation, the two groups turned out to be very different in many important parameters.

I have one small change that I would like to see in the manuscript. On page 4 of the Supplementary appendix (1.6) we are told that calf data suggested that 10 persons in each arm would give adequate power to this trial. We are not given a reference to the calf data; I think we should see that and the numerical data that went into the power calculation.

Author response: The calf data showing partial, limited efficacy of CFZ is in the process of being written up. Therefore, in the Supplementary Appendix we have clarified this as 'unpublished data, Michael Riggs, University of Arizona' (Supplementary Appendix, p4).

The lack of benefit from CFZ will be disappointing to many and the problems with this trial suggest that it won't be the last word on the subject, but the apparent dis-benefit of the drug on parasite excretion in the treatment group (although non-significant) will increase confidence in the author's conclusions.

Author response: Thank you, we agree.

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Author response: Subjects hospitalized with symptomatic diarrhea were managed by the clinical team and treated with ciprofloxacin, which is the standard of care. The stool TaqMan assay results were not performed in real-time and therefore results were not available until after conclusion of the study. We clarified this in the discussion (p15). Also it should be noted that the primary outcome was a change in cryptosporidium excretion, and not change in diarrhea. We have clarified this in the discussion also (p15).

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Author response: On L216-217 we stated that the CFZ group has “more pathogens detected at higher quantities (67% v. 30%), and clarified in the same paragraph (L330-334) the issue of GEMS diarrheagenic amounts. We have re-organized the paragraph so that the information is clearer (p10). However, please note the primary efficacy outcome was reduction in cryptosporidium shedding and not diarrhea resolution. We knew that it was likely that those infected with cryptosporidium would be also infected with other pathogens, and this might reduce the likelihood of efficacy in resolving diarrhea. But the results were very clear, cryptosporidium shedding was not reduced by clofazimine. We clarified this in the discussion (p15).

Line 326- This could be expressed more clearly- are the authors saying that the organism is exposed to the intraluminal CFZ and that efficacy may not be measured by plasma levels or that the organism itself is impairing absorption of the drug?

Author response: We have clarified the sentence to state that the parasite may not be well-exposed to intraluminal CFZ as it is located in a vacuole under the epithelial plasma membrane and faces in towards the gut lumen (p15).

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cryptosporidiosis in persons with HIV. It was a two-part study with 20 persons enrolled in a blinded, placebo-controlled RCT. Individuals randomized to active treatment with clofazimine worsened during the trial with a > 2 log (100-fold) increase in parasite excretion. 3 of 10 persons treated with CFZ died compared to 1 of 10 placebo treated individuals. In the second part of the report, the authors report the pharmacokinetics of CFZ in persons with HIV but without cryptosporidiosis. The report is well written.

Cryptosporidiosis in persons with HIV and low CD4 counts is often a lethal disease. No direct, primary therapy has been shown to be effective in persons with HIV. Nitazoxanide is of limited efficacy in persons without HIV, and of no proven benefit in persons with HIV. The core of treatment of cryptosporidiosis in persons with advanced HIV is prevention of death from dehydration and electrolyte disturbances while immune reconstitution is attempted with antiviral therapy. Many reports from the advent of the HIV pandemic described the extremely short life span of persons with untreated cryptosporidiosis, often less than 2 weeks from the time of presentation.

Human subjects approval after IRB review was granted, and no ethical issues are apparent. Many SAEs, including death, occurred in this population, and one person who was treated with CFZ was reported by the site to have a medication-related SAE. However, this was not upheld in review by the monitoring committee.

Dosing of clofazimine for the clinical trial was based upon the 'maximum given in clinical practice' of 100 mgs thrice daily to adults > 50 kgs, or half that dose if < 50 kgs. In the second part of the report, participants were matched to the active arm of the first part of the trial based upon age, gender, and weight. Quantitative PCR was used to assess excretion of the parasite, using first-passed-stool of the morning. In addition, all stools were collected during an 8-hour periods during the 5 days of inpatient treatment. The spectrum of Cryptosporidium species detected in participants included C. parvum, C. hominis, C. meleagridis, the unusual species C. viatorum, and 3 of unknown species.

Between 18 December 2017 and 14 February 2019, 5,790 persons were assessed for eligibility. 494 were prescreened for Cryptosporidium in feces. 67 were positive and 22 were randomized to CFZ or placebo. Although 12 were randomized to CFZ, only 10 completed treatment, and 1 more person withdrew from the study during the outpatient phase. Despite randomization, the active CFZ group was more male, had a lower BMI, and indications of more serious infection with greater stool output weight, more enteropathogens detected, and more advanced HIV (CD4 mean was ~ 25 compared to ~ 170 in the placebo group). Of importance, both the placebo and the active treatment groups had high HIV viral loads indicating that their HIV antiviral therapy was not effective.

Author response: We agree, we made these points in the body of the paper and have now added these points to the abstract (p3-4).

In the CFZ and placebo groups, there was no significant change in stool weight, frequency, consistency, or diarrhea grade during observation. In contrast, in the CFZ group, CR shedding increased by two orders of magnitude (when measured per gram) or in total calculated shedding (one order of magnitude). The graphs provided in the manuscript are
Persons with cryptosporidiosis (Part A) had serum levels of CFZ that were about half of those in persons without cryptosporidiosis (Part B) treated in the second phase of the report.
The authors report that plasma levels of HIV drugs in Part A subjects were detected at "similar" levels to Part B subjects suggesting they were compliant with their first-line ARV therapy, and that ARV resistance "might be driving HIV treatment failure." This suggests that the research team did not consider detecting, and addressing, ARV resistance in the study protocol.

Author response: In planning this single center study, our preliminary data to inform the design of the clinical trial did not include lab diagnostics, since standard lab tests such as full blood count and comprehensive metabolic profiles are not always available nor routinely done for patients hospitalized with diarrhea in Malawi, a setting with limited resources. HIV care in Malawi, as evidenced by national guidelines, and clinical care in

general, is guided by clinical presentation rather than by laboratory values. Therefore, the research team was not aware of the extent of laboratory abnormalities that were subsequently detected, including ARV resistance, among this patient population. When HIV viral load results eventually became available, these results were reviewed by a clinician and subjects were contacted and referred to HIV clinic to switch to second-line ARV regimens. This is now stated in the discussion (p14).

This reviewer strongly objects to the data on HIV treatment and resistance not being more forwardly placed in this report. First, HIV viral loads in the active treatment, and placebo, groups were extremely high. Mean viral loads in the CFZ group were 2.4 x 105 and 6.8 x 105 in the placebo arm. This is prima facie evidence that the antiviral treatment the participants were taking were not active. The core of treatment of cryptosporidiosis in persons with HIV is the prevention of death while immune reconstitution with antivirals is put in place. The authors do not state if a clinician reviewed the CD4 count, and viral load data, and made the (elementary) assessment that their treatment for HIV had failed. The authors do not state if the presence of a life-threatening opportunistic infection prompted a review of the participant's medical therapy and a change in their HIV antivirals because of presumptive resistance. While this study was conducted in a resource-challenged developing country, these basic assessments of HIV treatment adequacy were available and easily interpretable.

Author response: We agree with the reviewer that this is an important point of the paper, and we have tried hard in the revision to bring out this point. To bring it forward even more, we have added these issues to the abstract (p3-4). As stated above, one of the challenges of conducting a clinical trial such as this in a low-resource setting is that the full extent of health status of these patients did not become apparent until diagnostic testing was provided as a part of this trial. The research team did review all laboratory results, including CD4 count and viral load, and followed up with subjects in person to communicate these results to them and also to refer them to HIV clinic if warranted. We have revised the discussion to address these concerns (p14).

This report clarifies that clofazimine, at the doses administered, achieved a serum level in persons with ineffective antiviral therapy that was half that of persons with well-controlled HIV. It clarifies that clofazimine had no evident positive effect in persons with essentially untreated HIV. The mean viral load for participants in the Part B portion of the study was 2.6 x 102, a thousandth that of the persons who received clofazimine in the Part A portion of the study. Three-fold differences in viral loads are considered of clinical significance.

Key questions about this study are entangled around the inadequacy of treatment for HIV. It is possible that clofazimine, when administered to persons whose HIV is well treated (as demonstrated by a low viral load), might have a therapeutic effect. The fact that serum levels in persons with well-suppressed HIV were twice as high suggests that in the setting of Cryptosporidium infection, the drug was not as well absorbed. This would not be surprising given the architectural and functional changes seen in persons with active cryptosporidiosis.

Author response: We have added these points in the discussion (p16).

The authors note that the parasite replicates within a parasitophorous vacuole which may be difficult to drive CFZ into. It is no doubt all the more difficult to achieve CFZ levels in such a location in the range desired when baseline absorption is poor. An intravenous form of clofazimine was described in the past and the authors do not discuss whether or not such a formulation would have been a better choice in this pilot study. It
could be argued that in this clinical setting, oral and not iv therapy is appropriate to study, but this reviewer believes the authors must address this issue.

Author response: Intravenous CFZ was not a formulation offered by our supplier and therefore was not considered for this trial. In addition, an intravenous preparation of CFZ would not be available to repurpose for use in outpatients infected with cryptosporidium. We have included these points in the discussion (p16).

	We thank you for your consideration. Yours sincerely, Pui-Ying Iroh Tam, MD, FAAP, FPIDS, FIDSA Site Principal Investigator, CRYPTOFAZ Head, Paediatrics and Child Health Research Group, Malawi-Liverpool Wellcome Trust

William A. Petri, MD
Associate Editor, *Clinical Infectious Diseases*

5 March 2020

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Key questions about this study are entangled around the inadequacy of treatment for HIV. It is possible that clofazimine, when administered to persons whose HIV is well treated (as demonstrated by a low viral load), might have a therapeutic effect. The fact that serum levels in persons with well-suppressed HIV were twice as high suggests that in the setting of Cryptosporidium infection, the drug was not as well absorbed. This would not be surprising given the architectural and functional changes seen in persons with active cryptosporidiosis.

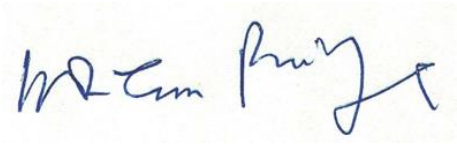
Author response: We have added these points in the discussion (p16).

The authors note that the parasite replicates within a parasitophorous vacuole which may be difficult to drive CFZ into. It is no doubt all the more difficult to achieve CFZ levels in such a location in the range desired when baseline absorption is poor. An intravenous form of clofazimine was described in the past and the authors do not discuss whether or not such a formulation would have been a better choice in this pilot study. It could be argued that in this clinical setting, oral and not iv therapy is appropriate to study, but this reviewer believes the authors must address this issue.

Author response: Intravenous CFZ was not a formulation offered by our supplier and therefore was not considered for this trial. In addition, an intravenous preparation of CFZ would not be available to repurpose for use in outpatients infected with cryptosporidium. We have included these points in the discussion (p16).

We thank you for your consideration.

Yours sincerely,



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Clofazimine for treatment of cryptosporidiosis in HIV-infected adults (CRYPTOFAZ): an experimental medicine, randomized, double-blind, placebo-controlled phase 2a trial

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Brief title: Clofazimine trial for cryptosporidiosis

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Key points

We evaluated clofazimine for treatment of adult HIV subjects with cryptosporidiosis.

Clofazimine was well tolerated, but did not reduce *Cryptosporidium* excretion or diarrhea compared with subjects treated with placebo. This trial forms a blueprint for future cryptosporidiosis therapeutic trials.

Abstract

Background: We evaluated efficacy, pharmacokinetics (PK), and safety of clofazimine (CFZ) in HIV-infected patients with cryptosporidiosis.

Methods: We performed a randomized, double-blind, placebo-controlled study. Primary outcomes in Part A were reduction in *Cryptosporidium* shedding, safety, and PK. Primary analysis was according to protocol (ATP). Part B of the study compared CFZ PK in matched HIV-infected individuals without cryptosporidiosis.

Results: Twenty Part A and 10 Part B participants completed the study ATP. Almost all Part A participants had high viral loads and low CD4 counts, consistent with failure of antiretroviral (ARV) therapy. At study entry, the Part A CFZ group had higher *Cryptosporidium* shedding, total stool weight, and more diarrheal episodes compared to the placebo group. Over the inpatient period, compared to those who received placebo, the CFZ group *Cryptosporidium* shedding increased by 2.17 $\log_2$ *Cryptosporidium* per gram stool (95% upper confidence limit: 3.82), total stool weight decreased by 45.3 g (p=0.37), and number of diarrheal episodes increased by 2.32 (p=0.87). The most frequent solicited adverse effects were diarrhea, abdominal pain, and malaise. Three CFZ and 1 placebo subjects died during the study. Plasma levels of CFZ in participants with cryptosporidiosis were 2-fold lower than Part B controls.

Conclusion: Our findings do not support the efficacy of CFZ for the treatment of cryptosporidiosis in a severely immunocompromised HIV population. However, this trial demonstrates a pathway to assess the therapeutic potential of drugs for cryptosporidiosis

treatment. Screening persons with HIV for diarrhea, and especially *Cryptosporidium* infection, may identify those failing ARV therapy.

250 words

Keywords: Cryptosporidium, diarrhea, HIV, therapeutic, trial

Introduction

Cryptosporidium infection and diarrhea (cryptosporidiosis) is a life-threatening infection in persons with HIV and also in young children in the developing world [1]. In children, cryptosporidiosis causes severe diarrhea [2], malabsorption and intestinal injury [3], excess mortality [2, 4], stunting and is associated with malnutrition [5]. There is a huge unmet need for *Cryptosporidium* drugs [6]: only nitazoxanide is licensed for treatment of cryptosporidiosis, but it has not shown any benefits as a treatment for HIV-infected and immunocompromised patients with cryptosporidiosis compared to placebo [7-9].

Clofazimine (CFZ), used for treatment of leprosy for more than 50 years, and currently part of treatment for multi-drug resistant TB, has recently been described as effective against *Cryptosporidium in vitro* [10]. The efficacy and pharmacokinetics (PK) of CFZ in HIV-infected patients with cryptosporidiosis are not known. We developed an experimental medicine study design to evaluate the safety, tolerability, PK and efficacy of CFZ in HIV-infected adults with cryptosporidiosis.

Methods

Study design and participants

The study was a single center, randomized, double-blind, placebo-controlled Phase 2a two-part study at Queen Elizabeth Central Hospital in Blantyre, Malawi. Participants were eligible for Part A if they were HIV-infected, aged 18-65 years, weight over 35·4 kg, on antiretrovirals (ARV) for at least 1 month, and with diarrhea duration of minimum 14 days. Participants for Part

B were HIV-infected without diarrhea or *Cryptosporidium*, and met none of the exclusion criteria. Full criteria are listed in the Supplementary Appendix. The study protocol was approved by the relevant regulatory and ethics committees before study initiation [11]. Participants provided written informed consent.

Study treatment and procedures

Part A participants were randomized 1:1 to receive either five days of oral CFZ or placebo, respectively (Figure 1). The dosage of CFZ administered was the maximum given in clinical practice, 100 mg three times daily if ≥50 kg or 50 mg three times daily for subjects <50 kg [12]. Participants for Part B were matched 1:1 to the first ten Part A subjects based on age (±5 years), gender, and weight (≥ or <50 kg; Supplementary Appendix).

We used a rapid diagnostic test (RDT) for *Cryptosporidium* screening (prototype immunochromatographic test strip for detecting *Cryptosporidium*, TechLabs Inc., Blacksburg, VA, USA) and an ELISA stool test (CRYPTOSPORIDIUM II™, TechLabs Inc.) for assessing *Cryptosporidium* shedding in serial stools during the trial. All *Cryptosporidium* shedding was confirmed and measured by qPCR, with a positive result being a cycle threshold (Ct) <35. The first collected stool of the day was obtained throughout the dosing and follow-up periods, for testing of the *Cryptosporidium* ELISA signal, as well as for measurement of *Cryptosporidium* shedding by qPCR. In addition, all stools were collected and pooled in 8-hour intervals during the inpatient phase of the study, Days -1 to 5 of dosing. Thus, total *Cryptosporidium* stool excretion was measured by qPCR during this time.

Stool enteropathogens present at baseline in addition to *Cryptosporidium* were detected using qPCR in a TaqMan Array Card (TAC, Thermo Fisher, Waltham, MA, USA) using a custom design developed at the Houpt Laboratory (Charlottesville, VA, USA; Supplementary Appendix) [13]. Measurements of anti-retroviral (ARV) levels in plasma and alteration after administration of CFZ were evaluated in the Van Voorhis/Arnold Laboratories (Seattle, WA, USA). Measurement of CFZ concentration in plasma and stool were performed at Q_2 Solutions (Ithaca, NY, USA).

After the 5-day inpatient study drug dosing, with daily clinical examination and laboratory sampling, all participants entered a 2 month follow-up period that included a visit 19-24 days post last dose, and a final visit 41-55 days post last dose. During each visit and with weekly phone calls, participants were monitored for safety and symptoms. Safety labs were repeated if there were any abnormalities previously. If participants could not be reached by phone, home visits were made.

Outcomes

There were two primary endpoints for Part A: the first was efficacy, assessed as reduction in the (log) number of *Cryptosporidium* shed in the first collected stool of each study dosing day of CFZ vs. placebo recipients in subjects treated according to protocol (ATP). The second primary endpoint was safety, including frequency and severity of solicited and unsolicited adverse events (AEs), serious adverse events (SAEs), adverse events of special interest and suspected, unexpected serious adverse reactions. Part B had two primary endpoints (CFZ in plasma, and total daily amount of CFZ eliminated in stool) to meet a single primary PK objective. Secondary

endpoints were the reduction in the (log_2) number of *Cryptosporidium* shed in stool compared to controls in the intention-to-treat (ITT) population, reduction in total daily *Cryptosporidium* shedding in those treated ATP, and as compared to controls in the ITT population, and reduction in severity of diarrhea over the study dosing period compared to controls.

An independent data safety monitoring board (DSMB) was involved in regular review of blinded safety data to monitor risks and benefits and to assess any potential safety issues arising during the study. Trial site monitoring of participant safety was carried out by the sponsor medical monitor, an independent local safety monitor, the contract research organization medical monitor, and overseen by the chief investigator (WVV). This study is registered with ClinicalTrials.gov, number NCT03341767.

Statistical analyses

As the Phase 2a study was exploratory, we initially planned an interim analysis after 20 subjects were randomized and treated ATP; this sample size was predicted to detect a therapeutic difference based on animal data from molecular endpoints. Due to slow enrollment, it was decided to convert the interim analysis to a final analysis (Supplementary Appendix).

The primary ATP analysis was performed using the randomized population who received at least 80% of scheduled doses, completed daily assessments of fecal shedding, and had no major protocol deviations. When missing data for the primary endpoint (log number of *Cryptosporidium* shed per gram stool) was not attributable to non-detectable *Cryptosporidium* (i.e. no stooling), multiple imputation was utilized (Supplementary Appendix).

The safety population consisted of all subjects that received at least one dose of study drug. The PK population consisted of all subjects who had at least one measurable PK concentration (Supplementary Appendix).

Due to the exploratory nature of the trial, no adjustments due to multiple testing were made; all statistical tests were performed with a one-sided alpha of 0.05. Statistical analyses were conducted using SAS version 9.3.

Results

Between 18 December 2017 and 14 February 2019, 5,790 adults were approached to assess eligibility. For randomization to CFZ vs. placebo (Part A), 494 were prescreened for *Cryptosporidium* presence in stool via RDT and qPCR, 67 participants were *Cryptosporidium* PCR-positive in stool and screened, and 22 were randomized (12 to CFZ and 10 to placebo, ITT group; Figure 1). Twenty subjects completed inpatient dosing ATP. There was one voluntary withdrawal (CFZ group) during the outpatient phase. There was no loss to follow-up.

The RDT and ELISA stool test had low sensitivity (41% for both) to identify participants and follow the presence/absence of *Cryptosporidium* over time, compared with qPCR. The *Cryptosporidium* spp. identified were *C. parvum* (11/22, 50%), *C. meleagridis* (4/22, 18%), *C. hominis (*3/22, 14%), *C. viatorum* (1/22, 5%) and 3 unknowns. Coinfection of stool with multiple diarrhea enteropathogens was common, with a median of 4 co-pathogens (excluding *Cryptosporidium*) per subject (range 1-8). The most frequently identified co-pathogen was

enteroaggregative *E. coli* (64%), followed by *Shigella* toxin-positive enterotoxigenic *E. coli* (41%) and *Shigella*/enteroinvasive *E. coli* (23%). The baseline characteristics of participants are listed in Table 1. Despite randomization, compared to the placebo group the CFZ group had by chance: more males (67% vs. 20%), lower body mass index (16.3±1.7 vs. 18.0±3.1 kg/m^2), increased diarrhea output total stool weight (320.3±214.6 vs. 245.8±299.4 g), more pathogens detected at a diarrheagenic amount per Global Enteric Multicenter Study (GEMS) criteria (67% vs. 30%) [14], more advanced HIV immunosuppression (CD4 counts 25.3±24.4 vs. 170.4±321.7 cells/μL), and higher prevalence of *C. parvum* detected (58% vs. 40%).

Findings were similar for both ATP and ITT populations (Supplementary Table 1), and the ATP efficacy results are reported here. Stool *Cryptosporidium* excretion was persistent among Part A subjects throughout observation (Supplementary Figures 1 and 2), even at 41-55 days after the last dose. There was no significant difference in *Cryptosporidium* shedding in the CFZ group compared to placebo (Figures 2A-B). There was a trend towards increased change-from-baseline in *Cryptosporidium* shedding in the first stool of the day in the CFZ-treated group vs. placebo, with a difference in means of 2.17 $\log_2$ *Cryptosporidium* per gram ([95% upper confidence limit (CL): 3.82]), and in total *Cryptosporidium* shedding with a difference of means of 1.02 $\log_2$ *Cryptosporidium* ([95% upper CL: 2.50]); the opposite result expected if CFZ was efficacious. There was no significant change in diarrhea in the CFZ group compared to placebo, whether measured by total stool weight change-from-baseline, number of diarrheal episodes, stool consistency grade, or severity diarrhea grade (Figures 2C-F).

For the PK of CFZ in HIV-infected subjects without diarrhea or *Cryptosporidium* (Part B), 92 were prescreened, 18 were screened, and 11 received CFZ, with one voluntary withdrawal during the inpatient phase. Part A subjects had about 2-fold less plasma exposure of CFZ than Part B subjects on day 5 (ratio AUC_{0-24}: 0.607), and on day 1 of the inpatient dosing (ratio AUC_{0-24}: 0.478; Table 2, Figure 3; stool PK profiles are listed in Supplementary Appendix and Supplementary Figure 3).

For safety, solicited AEs (Table 3) - expected in persons with diarrhea - were experienced by all subjects in both CFZ and placebo groups. There were higher numbers of solicited AEs experienced in the CFZ group for diarrhea (9 (75%) vs. 4 (40%) in placebo), abdominal pain (8 (67%) vs. 7 (70%) in placebo), and malaise (6 (50%) vs. 3 (30%) in placebo), and more severe solicited AEs in the CFZ group (2 (17%)) than the placebo group (0 (0%); Supplementary Figures 4 and 5). No Part B subject experienced any solicited AE. The number of unsolicited AEs (Supplementary Table 2) was highest in the CFZ group (13 vs. 12 in placebo and 3 in Part B); the number of subjects who experienced AEs with fatal outcome was also higher in the CFZ group (3 (25%) vs. 1 (10%) in placebo and none in Part B). None of the fatalities were judged by the study medical monitors and DSMB to be CFZ-related (Supplementary Appendix).

Discussion

This is the first randomized, double-blind, placebo-controlled Phase 2a trial to evaluate CFZ for treatment of cryptosporidiosis in HIV-infected adults. The trial demonstrated that CFZ had no significant impact on *Cryptosporidium* shedding of the parasite, or on diarrheal episodes, stool weight, and consistency, compared to placebo. Evaluation of *Cryptosporidium* shedding in the

first stool of the day provided similar data to total daily *Cryptosporidium* shedding. The drug is generally well-tolerated. Four patients died, three of whom received CFZ and the fourth placebo. This rate of death was consistent with our a priori estimates and each case was reviewed by the independent DSMB. CFZ achieved 2-fold less plasma exposure among Part A subjects with diarrhea vs. Part B subjects without diarrhea.

The trial did show that HIV-infected adults with ≥3 days of diarrhea consistently excreted *Cryptosporidium* in their stools, even when assayed up to 60 days after enrollment. This demonstrates that this population would be appropriate to study the antiparasitic benefit of anti-*Cryptosporidium* drugs that do not depend on the immune response.

The trial did not show a reduction in *Cryptosporidium* excretion in this population treated with CFZ vs. placebo. This was the case whether one compared the *Cryptosporidium* excretion by qPCR as determined by the concentration in the first stool of the day, or by determining the total *Cryptosporidium* excreted per day. In fact, there was a non-significant trend towards slightly increased *Cryptosporidium* shedding in the CFZ group vs. the placebo, which was most evident at day 2 of study drug dosing. The trend towards increased shedding may reflect the more ill status of the CFZ subjects at baseline, as documented in their enrollment labs and health status. With a median HIV CD4 count of 23.5 cells/mm^3 (IQR 11.75, 43.75) and viral load of 168,097.5 copies/mL (IQR 94,044, 643,812.3), the mortality rate of 18% in the trial likely reflects advanced disease in our Part A cohort as a whole.

Within our cohort, compared to placebo, the CFZ group had more deaths, SAEs, and severe solicited AEs. All subjects with cryptosporidiosis reported the solicited AEs expected with CFZ, such as diarrhea, abdominal pain, malaise and nausea. However, these solicited AEs were present at baseline in Part A subjects, as might be expected in this population with cryptosporidiosis, and were universal in both treatment groups. There tended to be less solicited AEs over time, which correlated with less severity in diarrhea during the hospital phase, and the severity of AEs tended to decrease over time. None of the Part B subjects exposed to the same dose of CFZ reported solicited AEs, and only 3 Part B subjects reported unsolicited AEs, and these were generally mild.

A previous clinical trial for cryptosporidiosis treatment identified multiple safety concerns related to the health status of participants. This Phase 1-2 trial of miltefosine to treat HIV-related cryptosporidiosis in Zambian adults with chronic diarrhea was terminated early due to high mortality, lack of efficacy and development of SAEs that were attributed to the extreme metabolic abnormalities already present in patients enrolled in the trial [15]. In our trial, subjects with cryptosporidiosis also presented with electrolyte abnormalities, most commonly hypokalemia that required correction, and some required corrective treatment through the trial. In addition, there was also a very high incidence of active TB in the HIV-infected screening population. Screening by chest x-ray was inadequate likely because dehydrated subjects often do not have an infiltrate until rehydrated. Screening of sputum by GeneXpert or gram stain also was inadequate due to inability of dehydrated subjects to produce sputum. All deaths in our study were reported prior to instituting urine LAM screening at baseline. Once urine LAM screening

297 was instituted [16], 43% of our otherwise eligible subjects subsequently tested positive by urine
298 LAM and were excluded.
299
300 Part A participants were extremely immunosuppressed. Most had CD4 counts <25 cells/μL and
301 high HIV viral loads. Plasma levels of HIV medicines were detected at similar levels to Part B
302 subjects (unpublished data), suggesting that these Part A subjects were compliant with first-line
303 ARV therapy and that ARV resistance might be driving HIV treatment failure. Therefore,
304 screening for diarrhea in this population, and especially for *Cryptosporidium*, delineated those
305 more at risk for TB and ARV failure.
306
307 The predominant *Cryptosporidium* species was *C. parvum* subtype family IIc anthroponotic
308 (10/11, 91% of those with *C. parvum*). This was unexpected, given that the majority of
309 *Cryptosporidium* species identified in the pediatric GEMS and adult studies were *C. hominis* [17-
310 20]. However, a high prevalence of *C. parvum* has been noted in HIV/AIDS patients in Ethiopia,
311 where 92/140 (66%) of HIV/AIDS patients were positive by PCR-RFLP [21]. As *C. parvum* has
312 been associated with prolonged diarrhea in HIV-positive persons more frequently than *C.*
313 *hominis* [17] the trial inclusion criteria may have selected for this species.
314
315 Multiple copathogens were observed in stool, which may have contributed to the diarrhea [3],
316 but patients with symptomatic diarrhea were routinely treated with ciprofloxacin as standard of
317 care. *Cryptosporidium* may have driven the diarrhea in at least 15 of 22 subjects in this trial, as it
318 was the pathogen with the lowest C_t value, and may have been the pathogen in the greatest
319 quantity shed in stool. After applying GEMS cutoffs, which use C_t counts to determine clinically

relevant diarrhea [14], only 7 *Cryptosporidium* samples met diarrheagenic cutoffs, and only 11 samples met diarrheagenic pathogen criteria. As GEMS data were based on children, the lack of correlation between C_t value and clinical diarrhea likely reflects the differences seen in an adult population with severe HIV immunosuppression with prolonged diarrhea.

Our PK data suggests that diarrhea and/or *Cryptosporidium* infection negatively impacts CFZ plasma exposure. Since efficacy is likely driven by CFZ levels in the parasite, which may not be well-exposed to intraluminal CFZ as it is located in a vacuole under the epithelial plasma membrane, and faces in towards the gut lumen [22], plasma levels may not reflect efficacy as it would for systemic infections. The fact that serum CFZ levels in persons with well-suppressed HIV were twice as high suggests that in the setting of *Cryptosporidium* infection, the drug was not well absorbed. We propose that lower levels of CFZ likely exist in the epithelium layer in the Part A subjects, as passage through the gastrointestinal epithelium is required for access to the plasma. These lower levels may have contributed to the failure of efficacy against *Cryptosporidium*. However, we used the maximum dosage of CFZ that is accepted as safe in this trial [12], therefore increasing the dosage to improve efficacy may not be feasible. An intravenous form of clofazimine, described in the past [23], may have provided better systemic delivery of the drug; however, this was not a formulation available at the time of the trial.

One of the limitations of the study was the small sample size. This led to slightly uneven randomization (12 vs. 10) based on block size. Also, imbalances in the baseline characteristics were noted in the Part A subjects CFZ vs. placebo groups, with the CFZ group being more ill at

baseline. One possible confounder was the presence of multiple co-pathogens in the stool, which could have influenced diarrhea resolution.

For the conduct of future human experimental trials of cryptosporidiosis in this population, this study suggests that: 1) the screening population should be evaluated for TB detection, through urine LAM, and for electrolyte disturbances, particularly hypokalemia; 2) use of stool RDT in screening and ELISA tests on serial stools is not as sensitive as qPCR, and that we need only use qPCR to enroll and follow participants for *Cryptosporidium* excretion over time; 3) following serial *Cryptosporidium* shedding by qPCR of the first stool of the day, rather than total stool collection, is probably sufficient to assess efficacy; 4) given the ill status of enrolled subjects, an inpatient trial is merited and AEs and deaths may complicate safety evaluation of new study drugs; and 5) future trials would need to be multisite given the slow recruitment rate. There was, until this trial, few placebo-controlled trials in adults [24-26] and limited data on how to test the drugs in Phase 2a. This trial shows that HIV-infected adults with cryptosporidiosis excrete the parasite consistently and thus the effects of treatment on excretion would be a feasible way to monitor for efficacy in *Cryptosporidium* therapy.

In conclusion, this is the first controlled clinical trial to assess the safety, efficacy, and PK of CFZ for treatment of cryptosporidiosis. Although CFZ does not show promise as a novel therapeutic for *Cryptosporidium* infection, future human studies can use an approach based on lessons learned in this trial to assess the therapeutic potential of drugs for treatment of cryptosporidiosis.

Word count (3,149)

Table 1. Baseline characteristics of participants

Table 2. Comparison of pharmacokinetic parameters in Part A and B subjects

Table 3. Summary of adverse events

Figure legends

Figure 1. Part A trial profile

ATP, according to protocol; ITT, intention to treat

[a]Subject died after completing visit.

[b]One subject withdrew during inpatient phase but provided final blood draw.

Figure 2. Treatment response in the according to protocol group:

A) Mean change from baseline (CFB) in log number of cryptosporidium shed in first collected stool over time

B) Mean CFB in total daily cryptosporidium shedding over time

C) Mean CFB in total stool weight over time

D) Mean number of diarrheal episodes over time

E) Proportion of most severe stool consistency grade by time

F) Proportion of most severe diarrhea grade by time

Figure 3. Mean plasma concentration of CFZ in plasma by time

Supplementary Appendix

Supplementary Methods

Supplementary Figure 2. Stool cryptosporidium shedding in: A) First stool of the day B) Total daily stooling

Supplementary Figure 3. Mean amount of CFZ in stool by timepoint

Supplementary Figure 4. Maximum severity of solicited symptoms

Supplementary Figure 5. Frequency of adverse events by organ class and: A) Severity B) Relationship to treatment

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Declaration of interests

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568 **Table 1.** Baseline characteristics of participants

Characteristic	Part A CFZ group (n=12)	Part A placebo group (n=10)	Part B CFZ group (n=11)
Age, years	39.8 (±7.8)	39.1 (±12.0)	44.1 (±9.6)
Male sex (%)	8 (67%)	2 (20%)	7 (64%)
BMI, kg/m^2	16.3 (±1.7)	18.0 (±3.1)	18.9 (±1.4)
Pulse rate, beats/min	90.9 (±12.4)	95.9 (±14.9)	78.1 (±6.7)
Systolic blood pressure, mmHg	99.3 (±15.0)	106.4 (±16.5)	116.5 (±11.8)
Diastolic blood pressure, mmHg	68,3 (±10.1)	71.2 (±8.8)	75.7 (±12.3)
Hemoglobin, g/dL	10.6 (±2.2)	10.8 (±2.8)	14.0 (±1.3)
Hematocrit, %	32.3 (±6.5)	32.6 (±8.7)	42.3 (±3.6)
White blood cells, $10^9/L$	2.9 (±1.4)	3.8 (±2.8)	5.0 (±1.7)
Neutrophils, $10^9/L$	1.6 (±0.9)	2.1 (±2.1)	2.5 (±1.2)
Lymphocytes, $10^9/L$	0.8 (±0.5)	1.1 (±0.7)	2.0 (±0.8)
CD4 absolute, cells/µL			
Mean (±SD)	25.3 (±24.4)	170.4 (±321.7)	422.0 (±231.3)
Median (IQR)	23.0 (8.0, 32.0)	22.5 (17.0, 86.0)	361.0 (216.0, 634.0)
HIV viral load, copies/µL	241,981.5 (±262,806.03)	679,025.13 (±929,116.49)	257.5 (±805.7)

ARV duration, days	1424 (±1547.6)	2011 (±1409.3)	1265 (±1810.3)
Blood urea nitrogen, mmol/L	4.9 (±2.5)	3.9 (±1.1)	3.8 (±1.0)
Creatinine, µmol/L	82.0 (±37.2)	56.0 (±15.9)	65.4 (±14.0)
Alanine aminotransferase, IU/L	34.0 (±20.3)	40.3 (±19.5)	38.9 (±21.4)
Aspartate aminotransferase, IU/L	50.6 (±16.4)	63.0 (±30.4)	50.7 (±18.3)
Electrocardiogram (ECG)			
Normal (%)	11 (92%)	10 (100%)	11 (100%)
Abnormal, not clinically significant (%)	1 (8%)	0 (0%)	0 (0%)
QTc interval, ms	421.7 (±14.2)	418.3 (±17.0)	409.7 (±21.6)
Cryptosporidium spp. (%)			
C. parvum	7 (58%)	4 (40%)	N/A
C. hominis	2 (17%)	1 (10%)	N/A
C. meleagridis	1 (8%)	3 (30%)	N/A
C. viatorum	1 (8%)	0 (0%)	N/A
Unknown[a]	1 (8%)	2 (20%)	N/A
Co-pathogens detected at diarrheagenic amount [14] (%)	8 (67%)	3 (30%)	N/A

Diarrhea duration,[b] days	17 (±7.6)	34 (±57)	N/A
Stool ELISA positivity (D1, %)	7 (58%)	2 (20%)	N/A
Log number of cryptosporidium shed in first collected stool of day, *Cryptosporidium* per gram stool (D-1)	13.9 (±2.7)	15.0 (±2.2)	N/A
Total daily cryptosporidium shedding, *Cryptosporidium* per gram stool (D-1)	22.3 (±2.9)	22.1 (±3.2)	N/A
Total stool weight, g (D-1)	320.3 (±214.6)	245.8 (±299.4)	N/A
Most severe diarrhea severity grade[c] (mild)	9 (75%)	3 (30%)	N/A
Stool consistency severity grade ≥3 (D-1, %)	9 (75%)	6 (67%)	N/A
Number of diarrheal episodes,[c] D1	1.3 (±1.1)	0.8 (±1.3)	N/A

569 ARV, antiretroviral therapy; BMI, body mass index; D, day; IQR, interquartile range; SD,

570 standard deviation

571 All values are mean (±SD) unless otherwise listed.

572 [a]Failed to amplify on sequencing of 18s and gp60.

[b]Subjects with diarrhea duration entries '>2 weeks' were treated as 21 days for calculations of summary statistics.

[c]Observed over the first 24-hour dosing interval after the first study dose.

Table 2. Comparison of pharmacokinetic parameters in Part A and B subjects

PK parameter		Part A (n=12)		Part B (n=11)	
		Mean (±SD)	% CV	Mean (±SD)	% CV
Day 1	C_{min} (ng/mL)	35.83 (±37.28)	323	74.74 (±24.51)	46
	C_{max} (ng/mL)	97.55 (±117.9)	195	193.3 (±93.50)	58
	T_{max} (h)	19.73 (±5.67)	-	14.776 (±7.537)	-
	AUC_{0-24} (ng.h/mL)	1364.0 (±1754.0)	219	2851.0 (±1256.0)	50
Day 5	C_{min} (ng/mL)	258.8 (±353.1)	187	455.8 (±221.5)	47
	C_{max} (ng/mL)	280.7 (±355.2)	173	514.1 (±202.0)	39
	T_{max} (h)	9.679 (±10.81)	-	6.683 (±3.765)	-
	AUC_{0-24} (ng.h/mL)	6863.0 (±8552.0)	172	11298.0 (±5580.0)	59
Summary	$t_{1/2}$ (h)[a]	336.5 (±84.71)	25	535.5 (±4.950)	1
	R_{AUC}	5.905 (±3.516)	57	4.111 (±1.579)	50

AUC, area under the curve; Cmax, peak plasma concentration; Cmin, trough plasma concentration; CV, coefficient of variation; R_{AUC}, accumulation ratio for AUC_{0-24} for Day 5 to Day 1; SD, standard deviation; Tmax, time to reach Cmax; $t_{1/2}$, elimination half-life

[a]Elimination half-life of clofazimine was previously found to be up to 70 days upon repeat dose administration [27]; therefore the relatively short plasma sampling schedule in this study may not be accurately capture the $t_{1/2}$ parameter in these populations.

607 **Table 3.** Summary of adverse events

		Part A – CFZ (n=12)	Part A – placebo (n=10)	Part B (n=11)
Any solicited adverse event	Any severity	12 (100%)	10 (100%)	0 (0%)
	Max severity	2 (17%)	0 (0%)	0 (0%)
Abdominal pain	Any severity	8 (67%)	7 (70%)	0 (0%)
	Max severity	1 (8%)	0 (0%)	0 (0%)
Vomiting	Any severity	4 (33%)	4 (40%)	0 (0%)
	Max severity	1 (8%)	0 (0%)	0 (0%)
Diarrhea	Any severity	9 (75%)	4 (40%)	0 (0%)
	Max severity	0 (0%)	0 (0%)	0 (0%)
Anorexia	Any severity	4 (33%)	3 (30%)	0 (0%)
	Max severity	0 (0%)	0 (0%)	0 (0%)
Skin discoloration	Any severity	0 (0%)	0 (0%)	0 (0%)
Nausea	Any severity	5 (42%)	5 (50%)	0 (0%)
	Max severity	1 (8%)	0 (0%)	0 (0%)
Malaise	Any severity	6 (50%)	3 (30%)	0 (0%)
	Max severity	1 (8%)	0 (0%)	0(0%)
Urgency of defecation	Any severity	5 (42%)	4 (40%)	0 (0%)
	Max severity	0 (0%)	0 (0%)	0 (0%)
Any adverse events with fatal outcome		3 (25%)	1 (10%)	0 (0%)

Number of unsolicited adverse events	13	12	3
Subjects with at least one unsolicited adverse event	6 (50%)	4 (40%)	3 (27%)
Subjects with a serious adverse event	5 (42%)	2 (20%)	0 (0%)
Any unsolicited adverse event related to study drug	2 (17%)	0 (0%)	3 (27%)
Any unsolicited adverse event leading to discontinuation of study drug	0 (0%)	1 (10%)	0 (0%)

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Figure 1

Click here to access/download;Figure (.tif and .eps files only);Figure 1-CONSORT DIAGRAM_v2.3_25Dec19.tiff

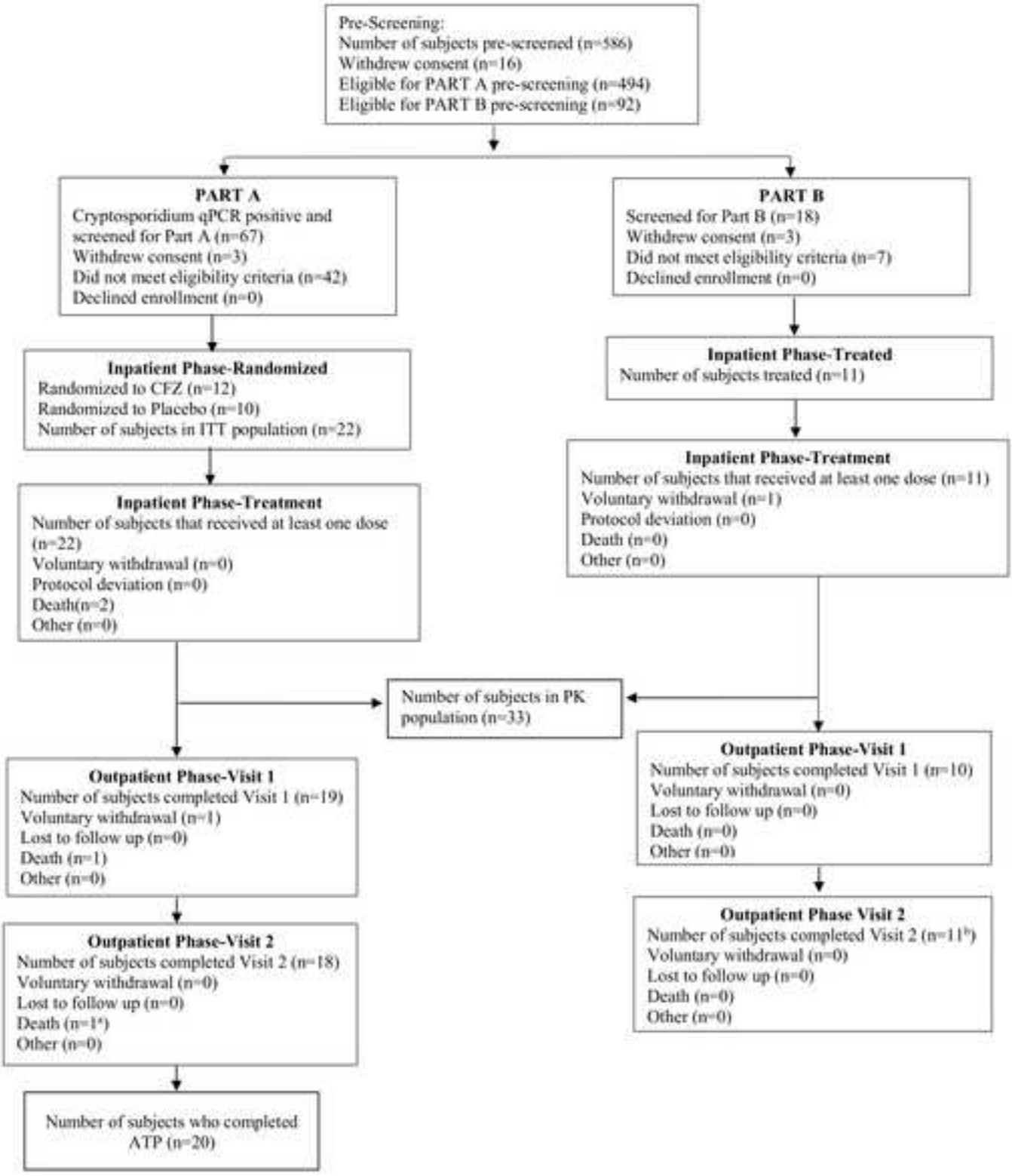


Figure 2

Click here to access/download;Figure (.tif and .eps files only);Figure 2_CFZ efficacy ATP_03Oct19.tiff

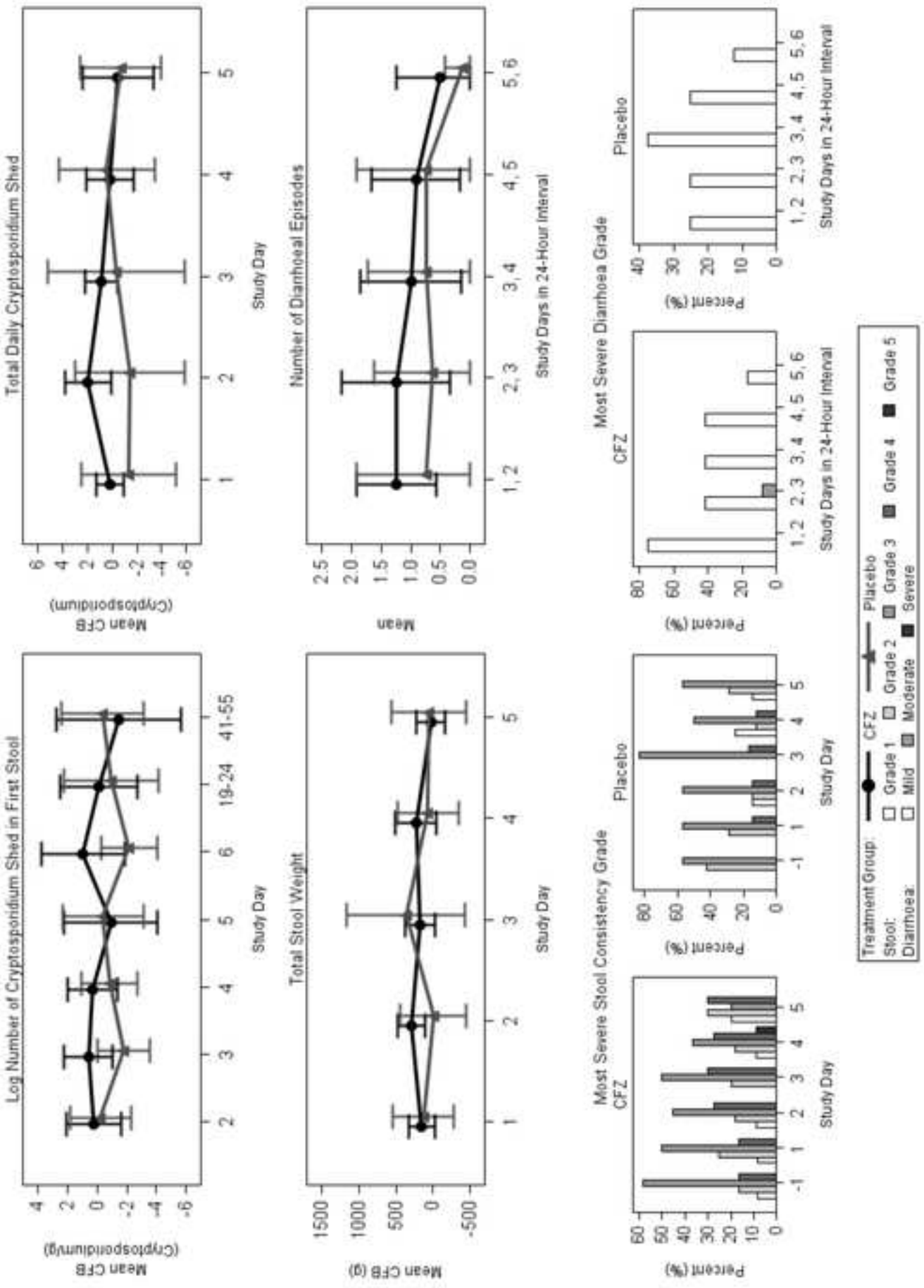
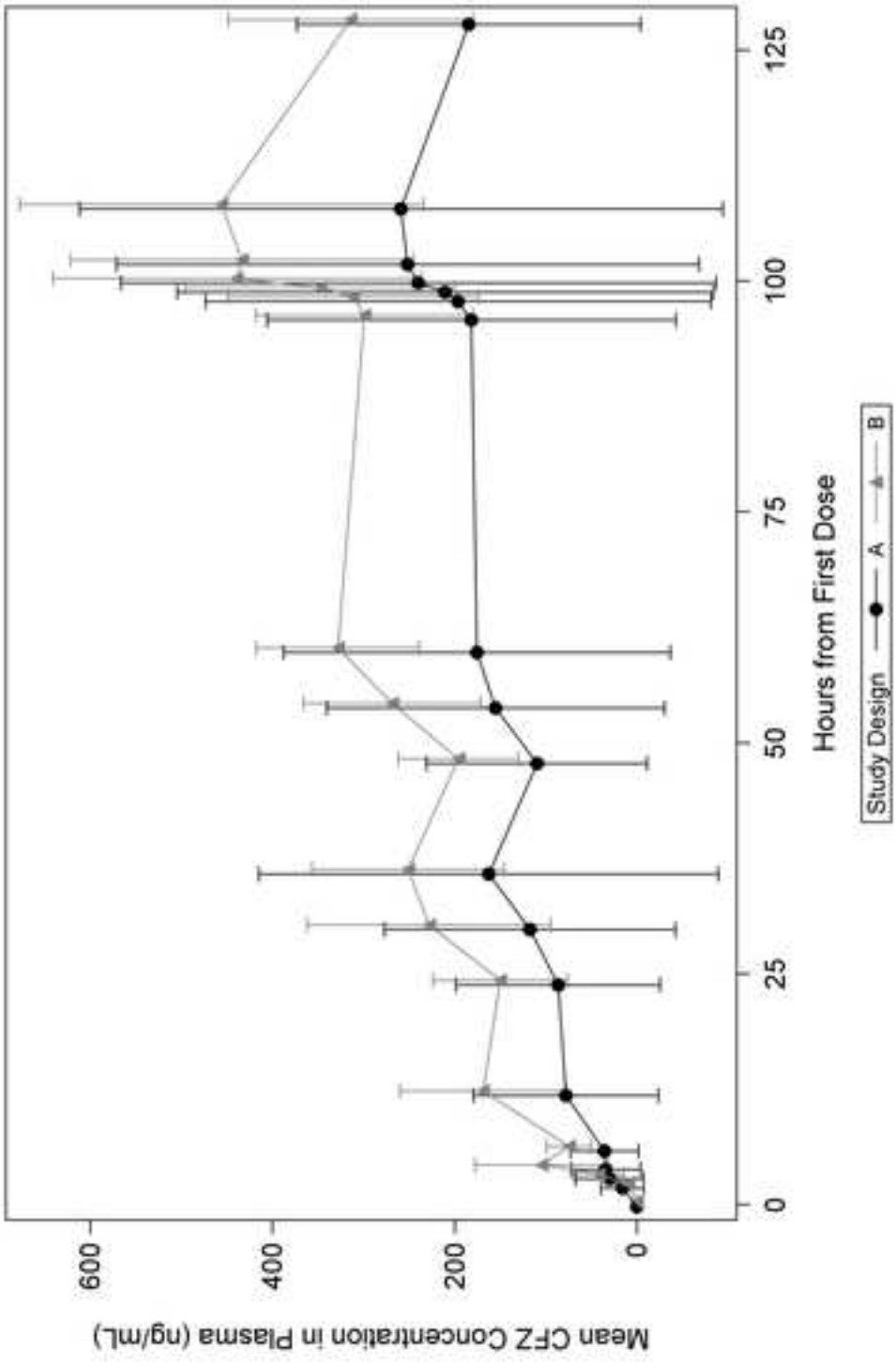


Figure 3

Click here to access/download;Figure (.tif and .eps files only);Figure 3_CFZ PK_26Sept19.tiff



Supplementary Appendix

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1. Methods

1.1 Study design

The study was a single center, randomized, double-blind, placebo-controlled Phase 2a two-part study at Queen Elizabeth Central Hospital in Blantyre, Malawi. Subjects were screened at this government, tertiary-level hospital, which serves the Southern region of the country, and were also referred from surrounding health centers within the Blantyre district. The study protocol and relevant supporting materials were approved by the National Health Sciences Research Committee (NHSRC) and the Pharmacy, Medicines, and Poisons Board in Malawi, and the Liverpool School of Tropical Medicine research ethics committee before study initiation.[1] Participants provided written informed consent. The NHSRC set participant compensation levels were used.

1.2 Participants

Participants were eligible for Part A if they met the following inclusion criteria: HIV-infected, aged 18-65 years, weight over 35·4 kg, on antiretrovirals (ARV) for at least 1 month, and with diarrhea duration of minimum 14 days. We estimated a priori a death rate in the HIV population in Malawi to be approximately 15%. Recruitment commenced on 18 December, 2017. On 6 April, 2018 after five subjects were randomized, eligibility criteria were amended to include participants with diarrhea duration of a minimum of 3 days and who have been on ARV for a minimum two weeks. Criteria were amended due to slow recruitment. Exclusion criteria included fever; evidence of active tuberculosis (by chest x-ray, sputum positive for TB by GeneXpert or Acid Fast Bacilli, and after 13 subjects were randomized, positive urine lipoarabinomannan (LAM)); history of allergy or hypersensitivity to CFZ; significant cardiac arrhythmia or ECG abnormalities; history of additional risk factors for Torsade de Pointes; family history of long QT syndrome; use of concomitant medications that markedly prolong the QT interval; pregnant and lactating women; use of systemic corticosteroids or anti-*Cryptosporidial* treatments within the preceding 28 days; and subjects with clinically significant laboratory value abnormalities at screening (hemoglobin <5 g/dL, serum potassium <3·0 mEq/L, and aspartate aminotransferase (AST) or alanine transaminase (ALT) ≥3 times upper limit of normal).

Participants for Part B were HIV-infected without diarrhea or *Cryptosporidium*, met none of the exclusion criteria, and were matched 1:1 to the first ten Part A subjects based on age (±5 years), gender, and weight (≥ or <50 kg).

1.3 Randomization and masking

We used a computer-generated randomization schedule where Part A group assignments of CFZ (Lamprene®, Novartis, Switzerland) and placebo were allocated in a 1:1 ratio, respectively, using a permuted block design with block size 4. Randomization was done by a contracted third-party contract research organization (CRO, Emmes, Rockville, MD, USA) that were involved in oversight but not the day-to-day clinical management of the study. The study drug and placebo were identical in appearance. Only the Emmes statisticians conducting the analysis and the pharmacists who prepared the pill packs were unmasked. The investigators, participants, and study site personnel involved in treating and assessing participants were masked to treatment allocation until the data was locked to further changes.

1.4 Procedures

Enrolled participants received five days of oral CFZ 50 mg three times daily for subjects <50 kg, or 100 mg three times daily if ≥50 kg, or placebo, respectively. Each dose was given half an hour after consumption of a fortified peanut-based paste (Plumpy Nut®, Nutriset, France). Participants were hospitalized for the five days of the study drug administration and returned on site for two follow-up visits. Laboratory testing was primarily carried out on-site at the Malawi-Liverpool Wellcome Trust Clinical Research Programme (MLW) laboratories. We used a rapid diagnostic test (RDT) for *Cryptosporidium* screening (prototype immunochromatographic test strip for detecting *Cryptosporidium*, TechLabs Inc., Blacksburg, VA, USA) and an ELISA stool test (CRYPTOSPORIDIUM II™, TechLabs Inc.) for quantifying *Cryptosporidium* shedding in serial stools during the trial. All *Cryptosporidium* shedding was confirmed and measured by qPCR, with a positive result being a cycle threshold (Ct) <35. The first collected stool of the day was obtained throughout the dosing and follow-up periods, for testing of the *Cryptosporidium* ELISA signal, as well as for measurement of *Cryptosporidium* shedding by qPCR. In addition, all stools were collected and pooled in 8-hour intervals during the inpatient phase of the study, Days -1 to 5 of dosing. Thus, total *Cryptosporidium* stool excretion was measured by qPCR during this time.

Stool enteropathogens present at baseline in addition to *Cryptosporidium* were detected using qPCR in a TaqMan Array Card (TAC, Thermo Fisher, Waltham, MA, USA) using a custom design developed at the Houpt Laboratory (Charlottesville, VA, USA).[2] TAC assays were performed at MLW, and also included previously published qPCR assays that distinguished *C. hominis* and *C. parvum*.[3] Further characterization of *Cryptosporidium* from baseline samples was achieved using Sanger sequencing targeting the 18S[4] and gp60 genes[5] performed at the Houpt Laboratory. The primer pairings originally described in Glaberman et al.[5] for the amplification of gp60 prior to Sanger sequencing were modified such that 5'-ATAGTCTCCGCTGTATTC-3' was paired with 5'-GGAAGGAACGATGTATCT-3' for the primary amplification and 5'-TCCGCTGTATTCTCAGCC-3' was paired with 5'-GCAGAGGAACCAGCATC-3' for the secondary nested amplification. Measurements of ARV levels in plasma and alteration after administration of CFZ were evaluated in the Van Voorhis/Arnold Laboratories (Seattle, WA, USA). Measurement of CFZ concentration in plasma and stool were performed at Q_2 Solutions (Ithaca, NY, USA) using liquid chromatography-tandem mass spectrometry (LC/MS/MS), which were validated for quantification of CFZ within the range of 1·0-1000 ng/mL in human plasma.

After study drug dosing, all participants entered a follow-up period of two months that included a follow-up visit within 19-24 days post last dose, and a final visit 41-55 days post last dose. During each follow-up visit and with weekly phone calls, participants were monitored for safety and symptoms. Blood and stool specimens were collected at each visit, and safety labs were repeated if there were any abnormalities previously. If participants could not be reached by phone, home visits were made.

In a resource-limited setting such as Malawi, laboratory investigations are not always available and therefore clinical care is primarily reliant on clinical presentation and symptoms. As part of the clinical care, laboratory results were reviewed by a clinician and subjects were referred for additional care as needed.

1.5 Outcomes

There were two primary endpoints for Part A, though formal statistical testing was only utilized for the primary efficacy endpoint. The first primary endpoint was efficacy, assessed

as reduction in the (log) number of *Cryptosporidium* shed in the first collected stool of each study dosing day of CFZ vs. placebo recipients in subjects treated according to protocol (ATP). The second primary endpoint was safety, based on safety assessments collected throughout dosing and follow-up periods, and consisted of frequency and severity of solicited and unsolicited adverse events (AEs) through study product administration, including serious adverse events (SAEs), adverse events of special interest (AESIs) and suspected, unexpected serious adverse reactions (SUSARs). Part B had two primary endpoints (CFZ in plasma, and total daily amount of CFZ eliminated in stool) to meet a single primary PK objective.

Secondary endpoints were the reduction in the (log_2) number of *Cryptosporidium* shed in stool compared to controls in the intention-to-treat (ITT) population, reduction in total daily *Cryptosporidium* shedding in those treated ATP, and as compared to controls in the ITT population, and reduction in severity of diarrhea over the study dosing period compared to controls.

1.6 Statistical analyses

Calf data on fecal shedding over time (unpublished data, Michael Riggs, University of Arizona) suggested that 10 individuals treated in each arm would be sufficient to give a >80% chance of seeing a difference with an efficacious drug. We were uncertain about the relevance of the animal data to the HIV subjects, and whether they would have consistent shedding over the period of treatment. Thus, we arbitrarily increased the sample size to 28 per group.

As the Phase 2a study was exploratory, we initially planned an interim analysis after 20 subjects were randomized and treated ATP. Due to slow enrollment, it was decided to convert the interim analysis to a final analysis.

Efficacy endpoints for Part A were summarized descriptively, and continuous efficacy variables were summarized at baseline and in terms of change from baseline at each day following study drug administration. The primary ATP analysis was performed using the randomized population who received at least 80% of scheduled doses, completed daily assessments of fecal shedding, and had no major protocol deviations. When missing data for the primary endpoint (log number of *Cryptosporidium* shed per gram stool) was not attributable to non-detectable *Cryptosporidium* (i.e. no stooling), multiple imputation was utilized. The fully conditional specification (FCS) method for arbitrary longitudinal missing data patterns was used to perform multiple imputation of the missing primary efficacy variable as well as any missing covariates. Mixed ANCOVA models for repeated measures were used to model and analyze the difference, between treatment groups, in the change from baseline in continuous endpoints over the inpatient period, and generally included baseline response, day, and treatment group as covariates. Gender and age were also included as covariates in models for the log number of cryptosporidium shed in the first collected stool (analyzed in the ATP and ITT populations). The day by treatment interaction term was considered for inclusion in models if statistically significant, and if included, the difference in efficacy measures in the last inpatient day was reported. Proportional odds models were used for the analysis of categorical endpoints (e.g., stool consistency and diarrhea severity). Upper 95% confidence intervals (CI) and p-values were derived from each model.

The safety population consisted of all subjects that received at least one dose of study drug. All safety analyses were descriptive. Ten subjects enrolled in Part B were matched to the first 10 subjects who completed Part A ATP, to develop a comparative description of the

absorption and excretion of the drug in the two groups. The PK population consisted of all subjects who had at least one measurable PK concentration. Plasma and stool drug concentrations were plotted at each timepoint for matched Part A and Part B subjects together, on linear scale. PK parameters were estimated through a non-compartmental analysis using Phoenix WinNonlin version 8.0 or later (Pharsight Corporation, Cary, NC, USA). The paired t-test was used to assess differences between groups for each PK parameter on days 1 and 5 (C_{min}, C_{max}, and AUC_{0-24}), and reported the geometric mean ratio between groups. We reported the Hodges-Lehmann estimator (pseudomedian) for the difference in each parameter between Part A and Part B subjects. P-values and 95% CI for each of the above tests were calculated.

Due to the exploratory nature of the trial, no adjustments due to multiple testing were made; all statistical tests were performed with a one-sided alpha of 0.05. Statistical analyses were conducted using SAS version 9.3.

To maximize the safety and integrity of the study, an independent data safety monitoring board (DSMB) was involved in regular review of blinded safety data to monitor risks and benefits and to assess any potential safety issues arising during the study. Trial site monitoring of participant safety was carried out by the sponsor medical monitor, an independent local safety monitor, the CRO medical monitor, and overseen by the chief investigator (WVV). This study is registered with ClinicalTrials.gov, number NCT03341767.

1.7 Role of the funding source
The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The first and last authors (PI, WVV) and the funders had full access to all the data in the study, following data lock. The first and last authors were responsible for the decision to submit for publication.

2. Results

2.1 Stool PK profiles
The total observed daily amount of CFZ eliminated in the feces on days 2 and 5 was not significantly different between Part A and B subjects (Supplementary Figure 3). Less than 2% of the cumulative CFZ doses was recovered in stool in both groups over the five days of stool collection.

2.2 Fatal outcomes
Two CFZ-treated subjects developed a fatal sepsis-like syndrome on day 5 of dosing. The first, judged to be unrelated to study drug, developed severe fatigue on the morning of the last dosing day with documented hypotension and was judged to have sepsis, received ceftriaxone and intravenous fluids, but rapidly died despite therapy. The second fatal case occurred in a subject who developed abdominal pains a day after receipt of CFZ, resolved when CFZ was stopped, then recurred when CFZ was restarted. An abdominal ultrasound demonstrated biliary stones, but a surgical consult could not be organized before the subject died of sepsis-like syndrome. The site judged the death to be related to CFZ administration, although the study medical monitors and DSMB judged this fatal SAE to be unrelated. The third CFZ-treated subject that died presented to the hospital with profound hypotension and diarrhea on day 18 after receipt of study drug. The patient did not respond to fluid resuscitation in the emergency suite and expired quickly after arrival. Death was attributed to the effects of chronic diarrhea, AIDS, and delayed presentation. The fatal SAE in the placebo group

occurred 47 days after study drug administration, in a subject who had been diagnosed with pulmonary and extrapulmonary TB after randomization with rehydration. The latter two deaths were judged not to be related to treatment. No autopsies were conducted in any of the deaths so exact causes of death could not be ascribed.

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Supplementary Table 1. Efficacy of clofazimine compared to placebo in the according to protocol (ATP) and intention-to-treat (ITT) populations

Outcomes	Difference in means	95% upper confidence limit	P-value
Parasitologic			
Change from baseline in log_2 number of cryptosporidium shed in first collected stool (log_2 *Cryptosporidium* per gram), ATP	2·17	3·82	0·984
Change from baseline in log_2 number of cryptosporidium shed in first collected stool (log_2 *Cryptosporidium* per gram), ITT	1·73	3·13	0·977
Change from baseline in total daily cryptosporidium shedding (log_2 *Cryptosporidium*), ATP	1·02	2·50	0·877
Change from baseline in total daily cryptosporidium shedding (log_2 *Cryptosporidium*), ITT	0·16	1·69	0·569
Diarrheal			
Change from baseline in total stool weight at Day 5 (g), ATP	132·05	314·48	0·888
Change from baseline in total stool weight at Day 5 (g), ITT	-45·30	179·88	0·366
Number of diarrheal episodes, ATP	1·92	5·73	0·802
Number of diarrheal episodes, ITT	2·32	5·74	0·871
Characteristics	Odds ratio		
Most severe stool consistency grade, ATP	0·66	10·39	0·401
Most severe stool consistency grade, ITT	1·85	26·26	0·651
Most severe diarrhea grade, ATP	5·33	29·28	0·947
Most severe diarrhea grade, ITT	4·87	23·85	0·950

Supplementary Table 2. Total number of unsolicited adverse events

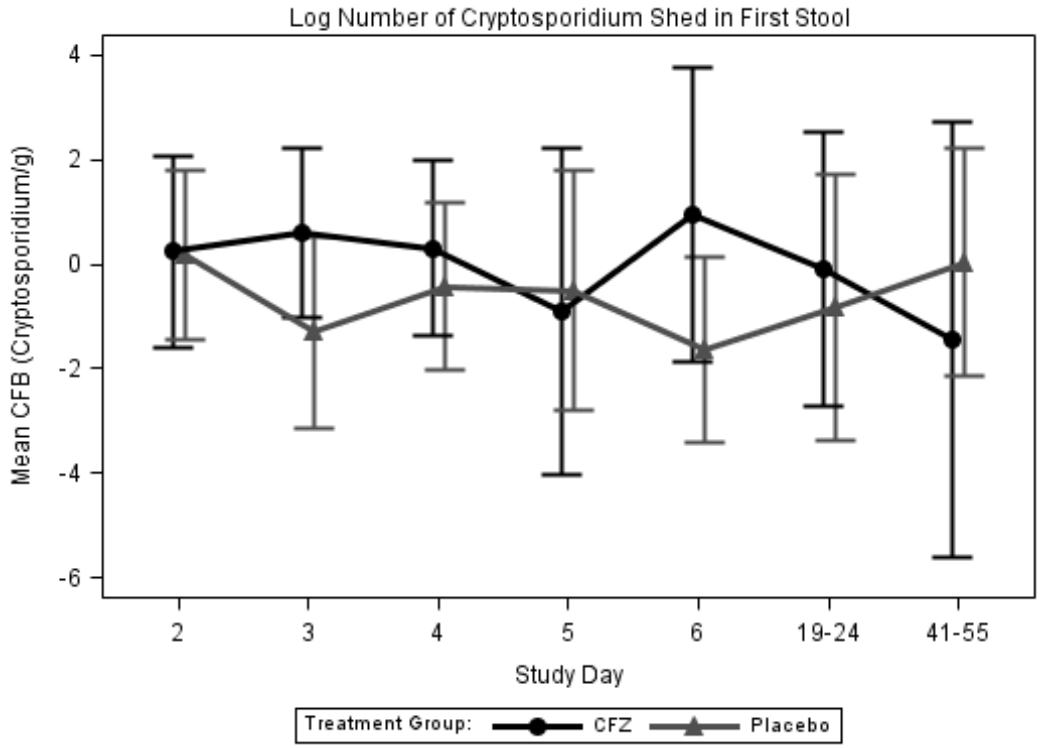
		Part A – CFZ (n=12)	Part A – placebo (n=10)	Part B (n=11)
MedDRA® system organ class	MedDRA® preferred term	No. of events	No. of events	No. of events
Any system organ class	Any preferred term	13	12	3
Blood and lymphatic system disorders	Anemia	0	3	0
Gastrointestinal disorders	Any preferred term	4	0	0
	Abdominal pain	1	0	0
	Anal fissure	1	0	0
	Diarrhea	2	0	0
General disorders and administration site conditions	Pyrexia	0	1	0
Infections and infestations	Any preferred term	4	6	0
	Extrapulmonary tuberculosis	0	1	0
	Gastroenteritis	1	1	0
	Lower respiratory tract infection	1	0	0
	Esophageal candidiasis	0	1	0
	Oral candidiasis	0	1	0
	Pneumonia	0	1	0
	Pulmonary tuberculosis	0	1	0
	Sepsis	1	0	0
	Septic shock	1	0	0
Investigations	Any preferred term	1	0	3
	Alanine aminotransferase increased	0	0	2
	Neutrophil count decreased	0	0	1
	White blood cell count decreased	1	0	0
Metabolism and nutrition disorders	Hypokalemia	1	1	0
Skin and subcutaneous tissue disorders	Decubitus ulcer	1	0	0
Vascular disorders	Any preferred term	2	1	0

	Hypotension	1	1	0
	Hypovolemic shock	1	0	0

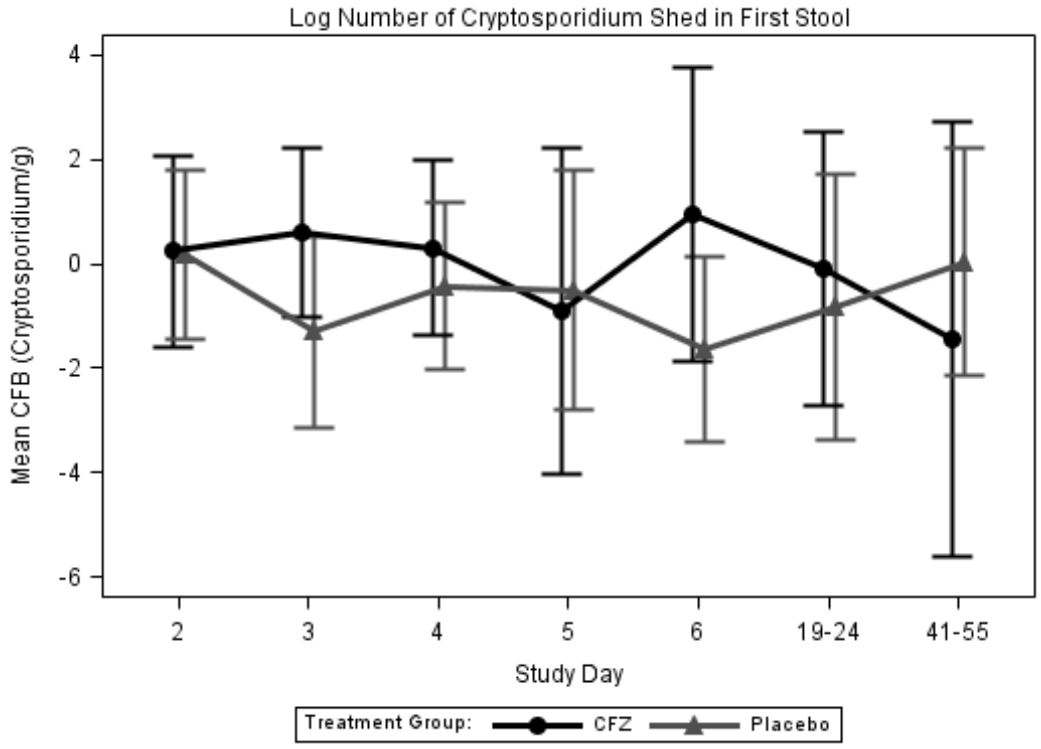
CFZ, clofazimine; MedDRA®, medical dictionary for regulatory activities

Supplementary Figure 1. Treatment response in the intention-to-treat group:

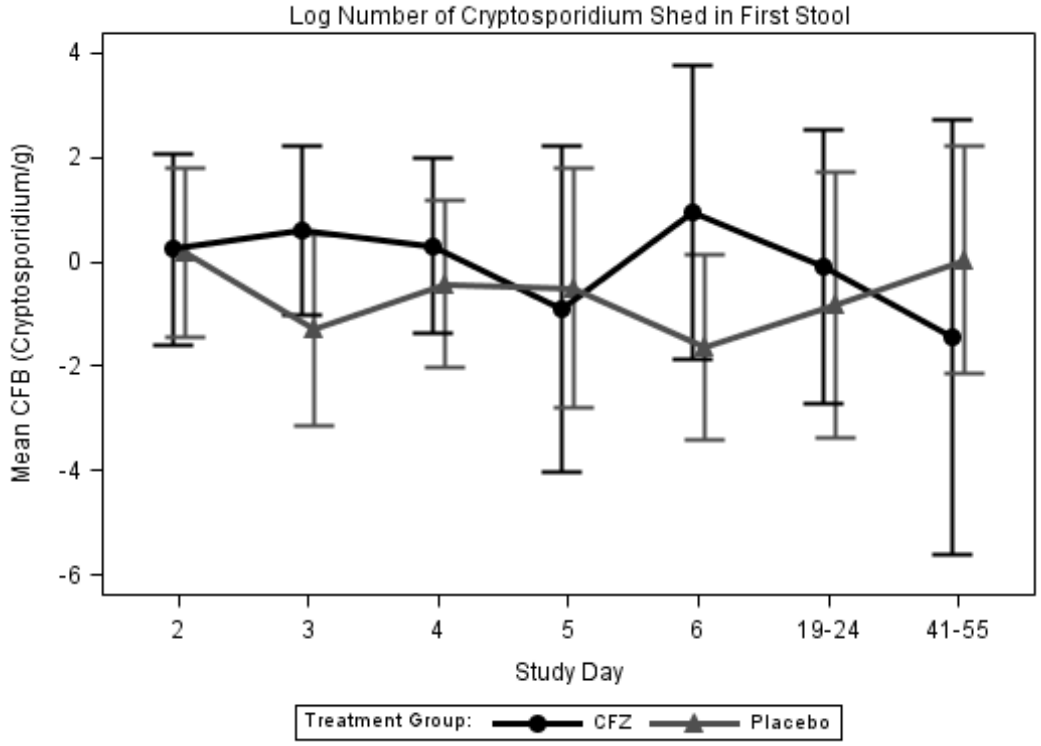
A) Mean change from baseline (CFB) in log_2 number of cryptosporidium shed in first collected stool over time



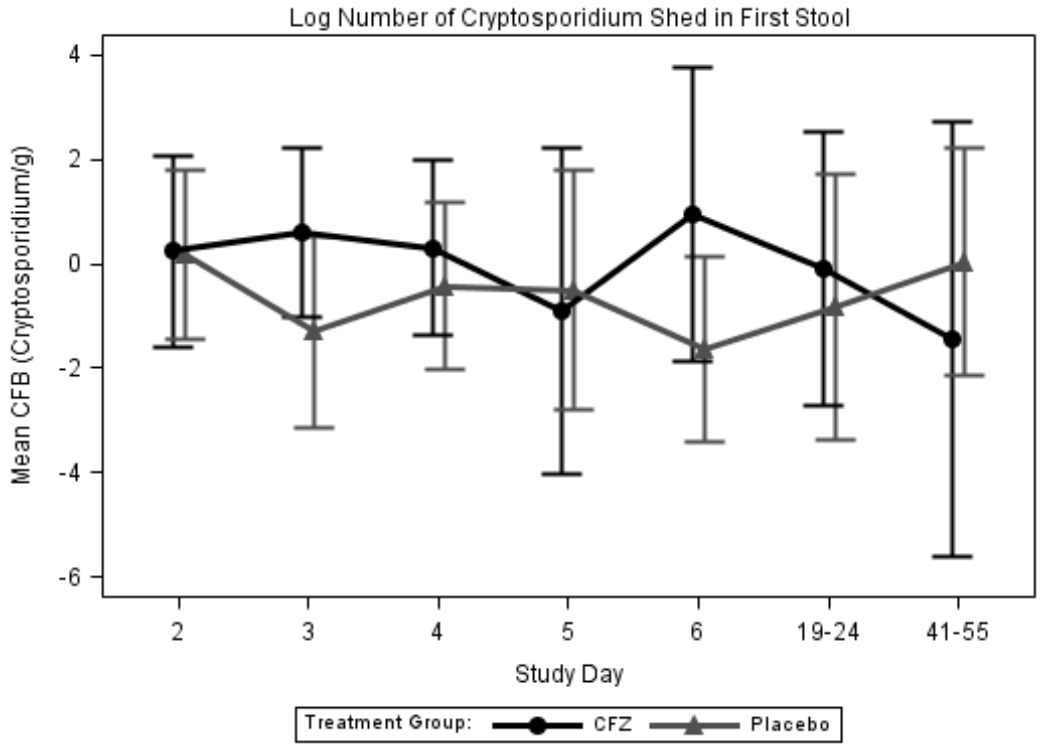
B) Mean CFB in total daily cryptosporidium shedding over time



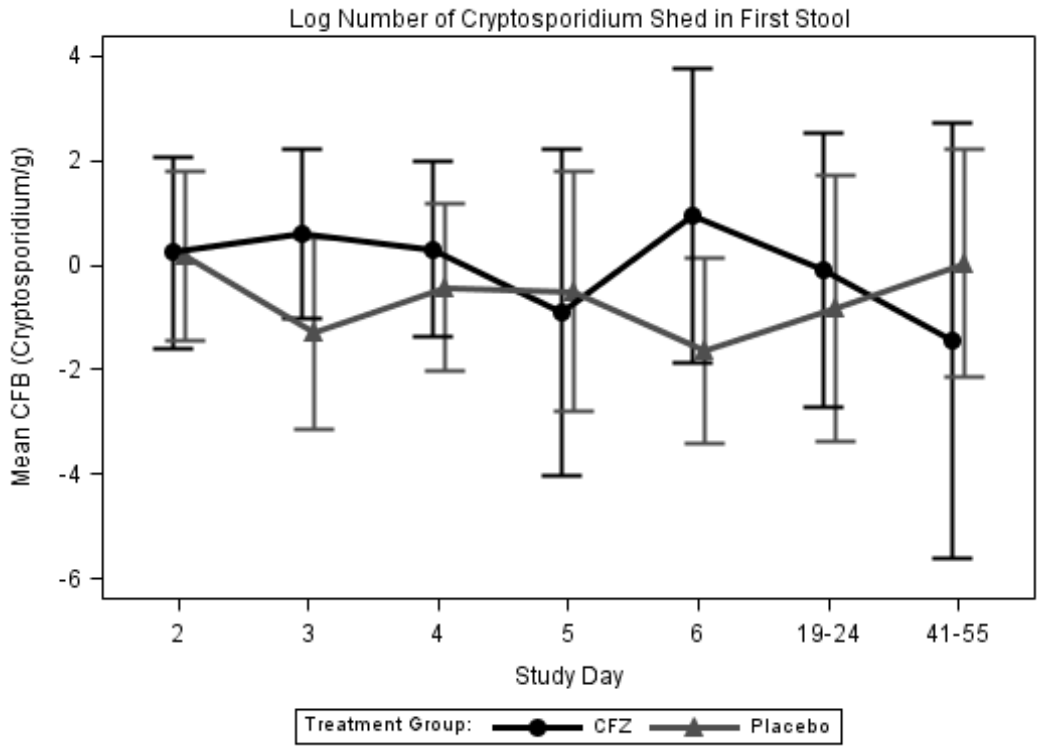
C) Mean total daily cryptosporidium shedding over time



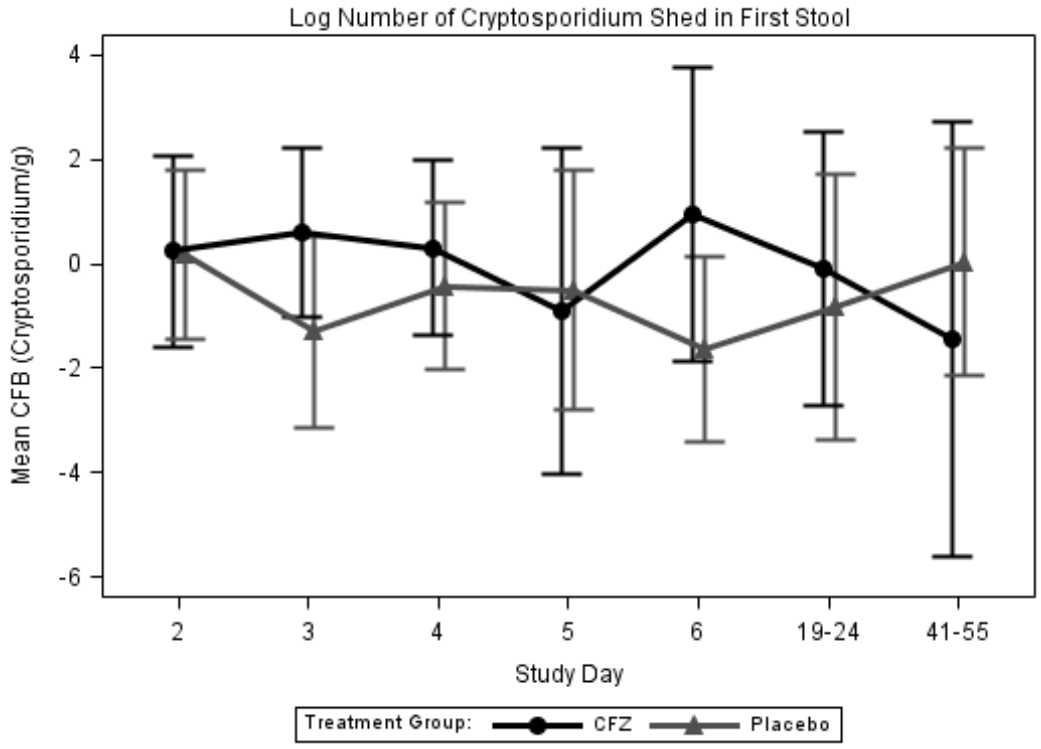
D) Mean CFB in total stool weight over time



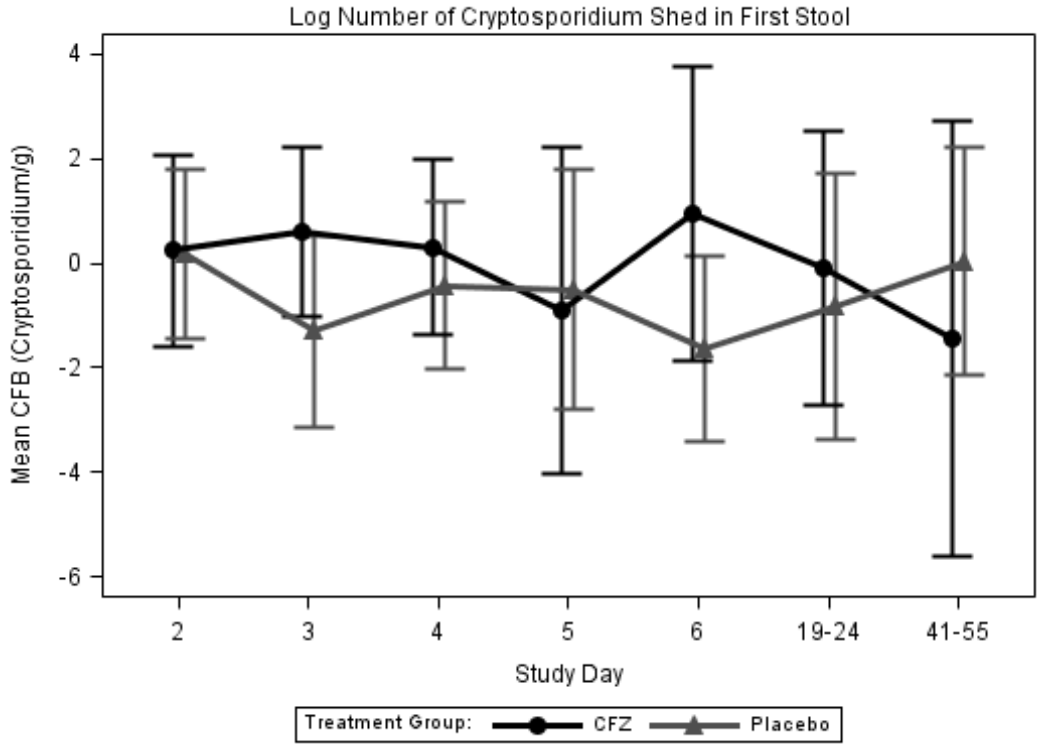
E) Mean number of diarrheal episodes over time



F) Proportion of most severe stool consistency grade by time

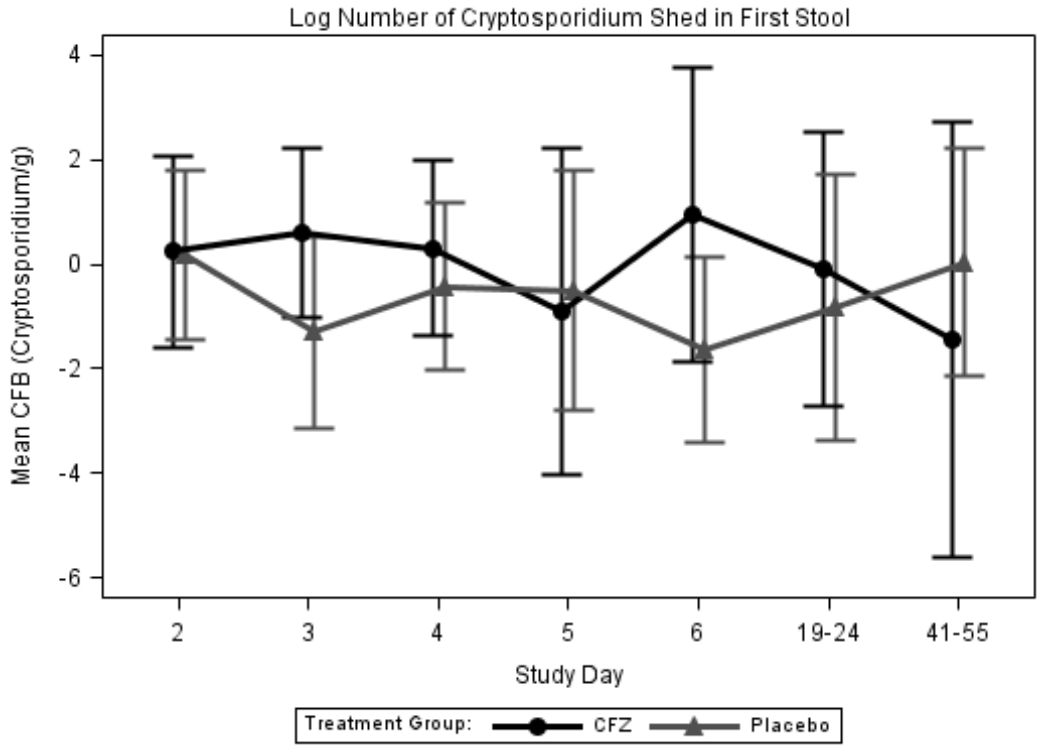


G) Proportion of most severe diarrhea grade by time

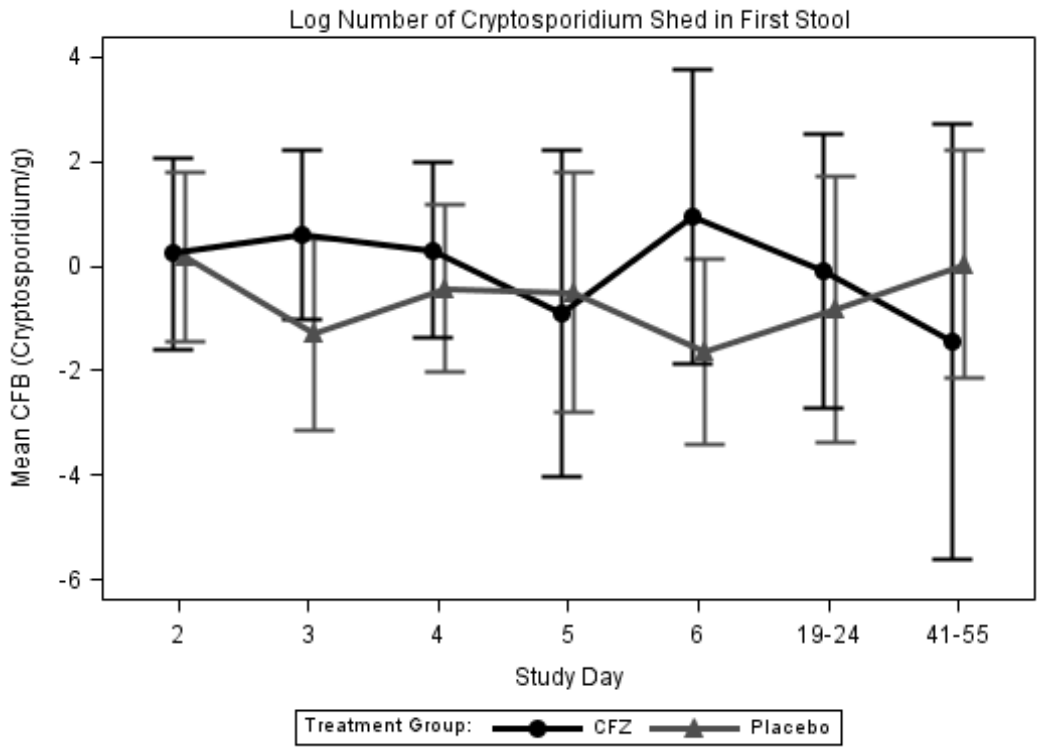


Supplementary Figure 2. Stool cryptosporidium shedding in:

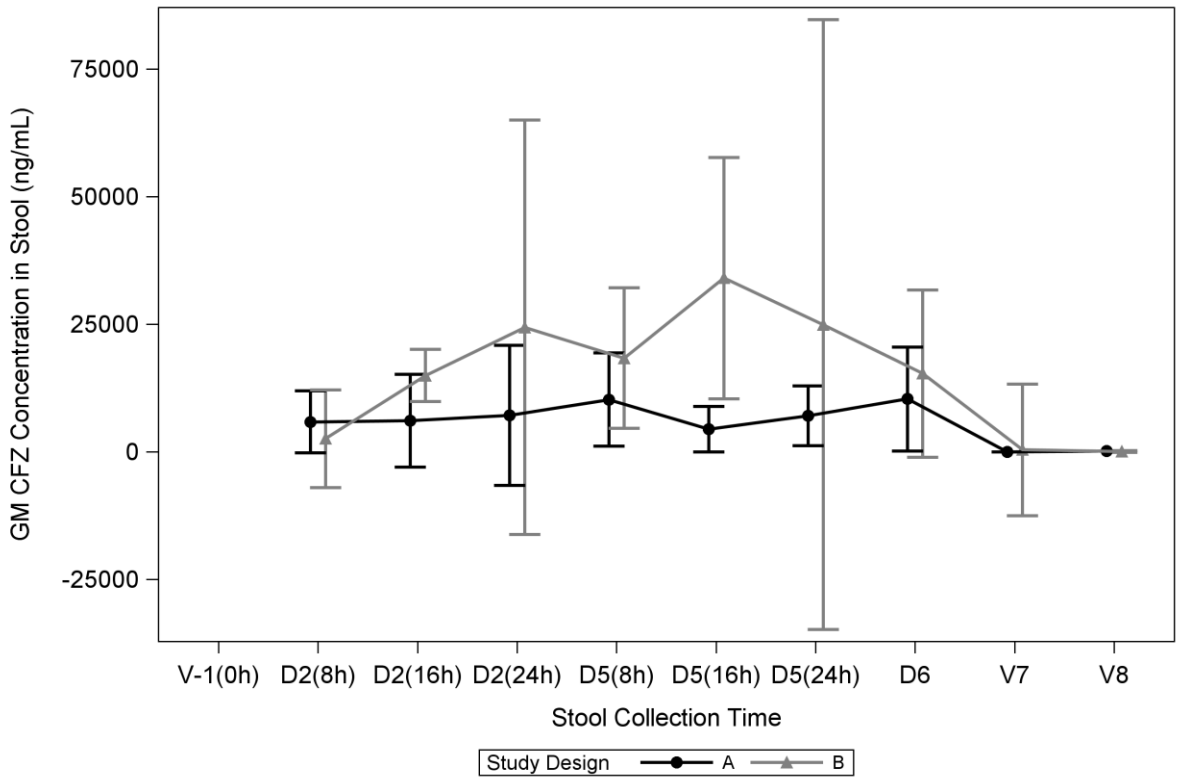
A) First stool of the day



B) Total daily stooling

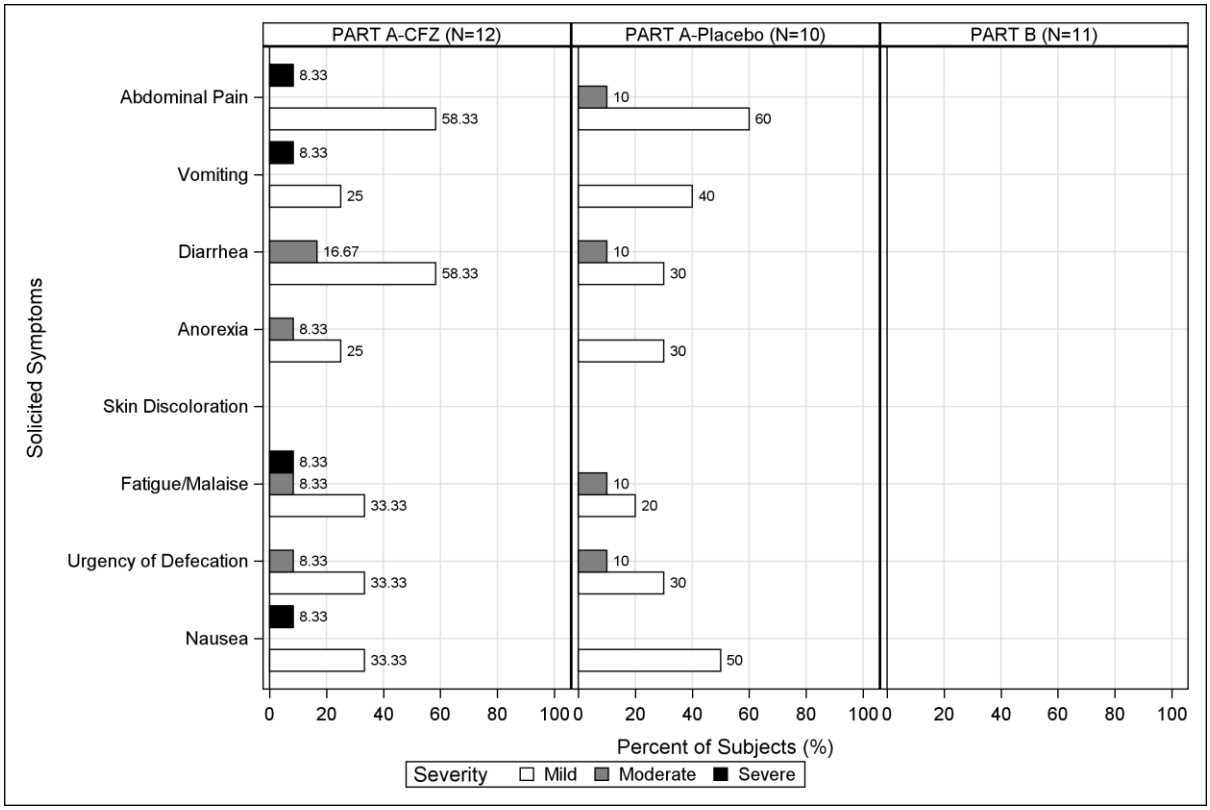


Supplementary Figure 3. Mean amount of CFZ in stool by timepoint



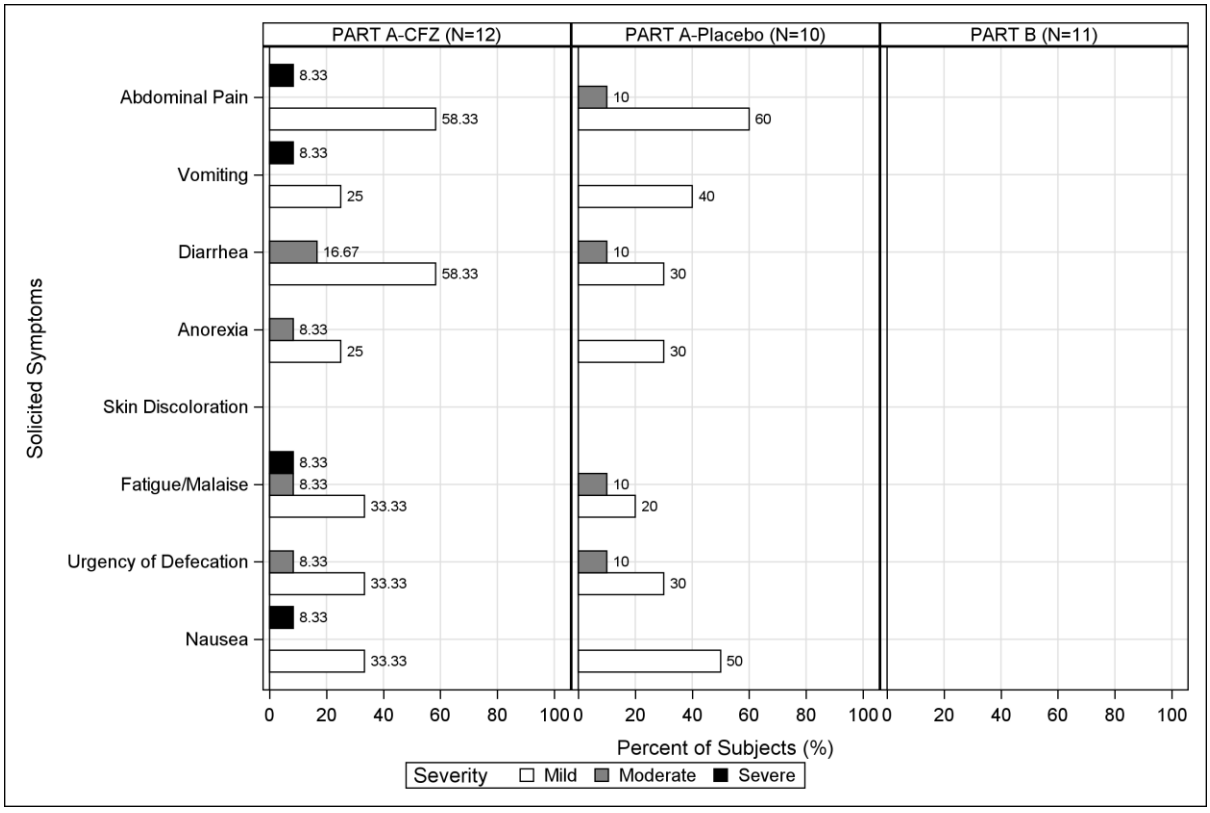
CFZ, clofazimine; D, day; GM, geometric mean; V, study visit

Supplementary Figure 4. Maximum severity of solicited symptoms

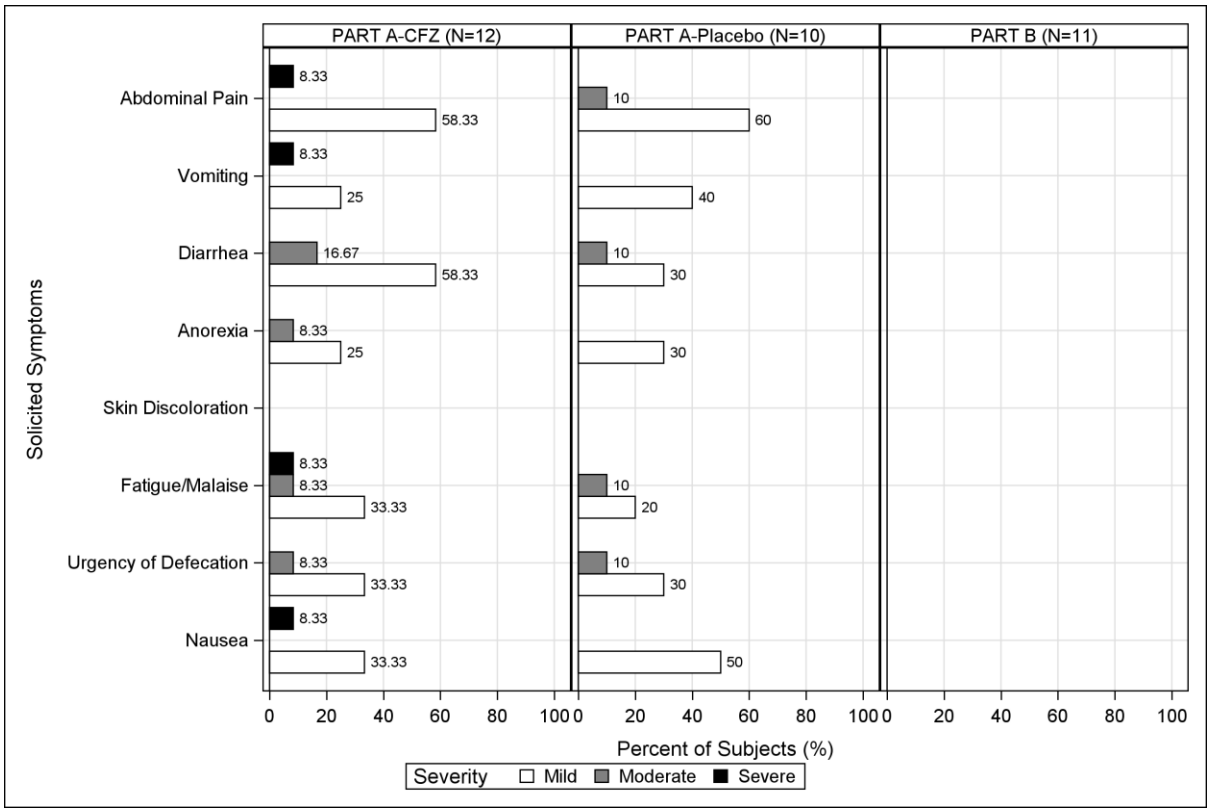


Supplementary Figure 5. Frequency of adverse events by organ class and:

A) Severity



B) Relationship to treatment



Supplementary Appendix

Table of contents

1. Methods

1.1 Study design

The study was a single center, randomized, double-blind, placebo-controlled Phase 2a two-part study at Queen Elizabeth Central Hospital in Blantyre, Malawi. Subjects were screened at this government, tertiary-level hospital, which serves the Southern region of the country, and were also referred from surrounding health centers within the Blantyre district. The study protocol and relevant supporting materials were approved by the National Health Sciences Research Committee (NHSRC) and the Pharmacy, Medicines, and Poisons Board in Malawi, and the Liverpool School of Tropical Medicine research ethics committee before study initiation.[1] Participants provided written informed consent. The NHSRC set participant compensation levels were used.

1.2 Participants

Participants were eligible for Part A if they met the following inclusion criteria: HIV-infected, aged 18-65 years, weight over 35·4 kg, on antiretrovirals (ARV) for at least 1 month, and with diarrhea duration of minimum 14 days. We estimated a priori a death rate in the HIV population in Malawi to be approximately 15%. Recruitment commenced on 18 December, 2017. On 6 April, 2018 after five subjects were randomized, eligibility criteria were amended to include participants with diarrhea duration of a minimum of 3 days and who have been on ARV for a minimum two weeks. Criteria were amended due to slow recruitment. Exclusion criteria included fever; evidence of active tuberculosis (by chest x-ray, sputum positive for TB by GeneXpert or Acid Fast Bacilli, and after 13 subjects were randomized, positive urine lipoarabinomannan (LAM)); history of allergy or hypersensitivity to CFZ; significant cardiac arrhythmia or ECG abnormalities; history of additional risk factors for Torsade de Pointes; family history of long QT syndrome; use of concomitant medications that markedly prolong the QT interval; pregnant and lactating women; use of systemic corticosteroids or anti-*Cryptosporidial* treatments within the preceding 28 days; and subjects with clinically significant laboratory value abnormalities at screening (hemoglobin <5 g/dL, serum potassium <3·0 mEq/L, and aspartate aminotransferase (AST) or alanine transaminase (ALT) ≥3 times upper limit of normal).

Participants for Part B were HIV-infected without diarrhea or *Cryptosporidium*, met none of the exclusion criteria, and were matched 1:1 to the first ten Part A subjects based on age (±5 years), gender, and weight (≥ or <50 kg).

1.3 Randomization and masking

We used a computer-generated randomization schedule where Part A group assignments of CFZ (Lamprene®, Novartis, Switzerland) and placebo were allocated in a 1:1 ratio, respectively, using a permuted block design with block size 4. Randomization was done by a contracted third-party contract research organization (CRO, Emmes, Rockville, MD, USA) that were involved in oversight but not the day-to-day clinical management of the study. The study drug and placebo were identical in appearance. Only the Emmes statisticians conducting the analysis and the pharmacists who prepared the pill packs were unmasked. The investigators, participants, and study site personnel involved in treating and assessing participants were masked to treatment allocation until the data was locked to further changes.

1.4 Procedures

Enrolled participants received five days of oral CFZ 50 mg three times daily for subjects <50 kg, or 100 mg three times daily if ≥50 kg, or placebo, respectively. Each dose was given half

an hour after consumption of a fortified peanut-based paste (Plumpy Nut®, Nutriset, France). Participants were hospitalized for the five days of the study drug administration and returned on site for two follow-up visits. Laboratory testing was primarily carried out on-site at the Malawi-Liverpool Wellcome Trust Clinical Research Programme (MLW) laboratories. We used a rapid diagnostic test (RDT) for *Cryptosporidium* screening (prototype immunochromatographic test strip for detecting *Cryptosporidium*, TechLabs Inc., Blacksburg, VA, USA) and an ELISA stool test (CRYPTOSPORIDIUM II™, TechLabs Inc.) for quantifying *Cryptosporidium* shedding in serial stools during the trial. All *Cryptosporidium* shedding was confirmed and measured by qPCR, with a positive result being a cycle threshold (Ct) <35. The first collected stool of the day was obtained throughout the dosing and follow-up periods, for testing of the *Cryptosporidium* ELISA signal, as well as for measurement of *Cryptosporidium* shedding by qPCR. In addition, all stools were collected and pooled in 8-hour intervals during the inpatient phase of the study, Days -1 to 5 of dosing. Thus, total *Cryptosporidium* stool excretion was measured by qPCR during this time.

Stool enteropathogens present at baseline in addition to *Cryptosporidium* were detected using qPCR in a TaqMan Array Card (TAC, Thermo Fisher, Waltham, MA, USA) using a custom design developed at the Houpt Laboratory (Charlottesville, VA, USA).[2] TAC assays were performed at MLW, and also included previously published qPCR assays that distinguished *C. hominis* and *C. parvum*.[3] Further characterization of *Cryptosporidium* from baseline samples was achieved using Sanger sequencing targeting the 18S[4] and gp60 genes[5] performed at the Houpt Laboratory. The primer pairings originally described in Glaberman et al.[5] for the amplification of gp60 prior to Sanger sequencing were modified such that 5'-ATAGTCTCCGCTGTATTC-3' was paired with 5'-GGAAGGAACGATGTATCT-3' for the primary amplification and 5'-TCCGCTGTATTCTCAGCC-3' was paired with 5'-GCAGAGGAACCAGCATC-3' for the secondary nested amplification. Measurements of ARV levels in plasma and alteration after administration of CFZ were evaluated in the Van Voorhis/Arnold Laboratories (Seattle, WA, USA). Measurement of CFZ concentration in plasma and stool were performed at Q_2 Solutions (Ithaca, NY, USA) using liquid chromatography-tandem mass spectrometry (LC/MS/MS), which were validated for quantification of CFZ within the range of 1·0-1000 ng/mL in human plasma.

After study drug dosing, all participants entered a follow-up period of two months that included a follow-up visit within 19-24 days post last dose, and a final visit 41-55 days post last dose. During each follow-up visit and with weekly phone calls, participants were monitored for safety and symptoms. Blood and stool specimens were collected at each visit, and safety labs were repeated if there were any abnormalities previously. If participants could not be reached by phone, home visits were made.

In a resource-limited setting such as Malawi, laboratory investigations are not always available and therefore clinical care is primarily reliant on clinical presentation and symptoms. As part of the clinical care, laboratory results were reviewed by a clinician and subjects were referred for additional care as needed.

1.5 Outcomes

There were two primary endpoints for Part A, though formal statistical testing was only utilized for the primary efficacy endpoint. The first primary endpoint was efficacy, assessed as reduction in the (log) number of *Cryptosporidium* shed in the first collected stool of each study dosing day of CFZ vs. placebo recipients in subjects treated according to protocol

(ATP). The second primary endpoint was safety, based on safety assessments collected throughout dosing and follow-up periods, and consisted of frequency and severity of solicited and unsolicited adverse events (AEs) through study product administration, including serious adverse events (SAEs), adverse events of special interest (AESIs) and suspected, unexpected serious adverse reactions (SUSARs). Part B had two primary endpoints (CFZ in plasma, and total daily amount of CFZ eliminated in stool) to meet a single primary PK objective.

Secondary endpoints were the reduction in the (log_2) number of *Cryptosporidium* shed in stool compared to controls in the intention-to-treat (ITT) population, reduction in total daily *Cryptosporidium* shedding in those treated ATP, and as compared to controls in the ITT population, and reduction in severity of diarrhea over the study dosing period compared to controls.

1.6 Statistical analyses

Calf data on fecal shedding over time (unpublished data, Michael Riggs, University of Arizona) suggested that 10 individuals treated in each arm would be sufficient to give a >80% chance of seeing a difference with an efficacious drug. We were uncertain about the relevance of the animal data to the HIV subjects, and whether they would have consistent shedding over the period of treatment. Thus, we arbitrarily increased the sample size to 28 per group.

As the Phase 2a study was exploratory, we initially planned an interim analysis after 20 subjects were randomized and treated ATP. Due to slow enrollment, it was decided to convert the interim analysis to a final analysis.

Efficacy endpoints for Part A were summarized descriptively, and continuous efficacy variables were summarized at baseline and in terms of change from baseline at each day following study drug administration. The primary ATP analysis was performed using the randomized population who received at least 80% of scheduled doses, completed daily assessments of fecal shedding, and had no major protocol deviations. When missing data for the primary endpoint (log number of *Cryptosporidium* shed per gram stool) was not attributable to non-detectable *Cryptosporidium* (i.e. no stooling), multiple imputation was utilized. The fully conditional specification (FCS) method for arbitrary longitudinal missing data patterns was used to perform multiple imputation of the missing primary efficacy variable as well as any missing covariates. Mixed ANCOVA models for repeated measures were used to model and analyze the difference, between treatment groups, in the change from baseline in continuous endpoints over the inpatient period, and generally included baseline response, day, and treatment group as covariates. Gender and age were also included as covariates in models for the log number of cryptosporidium shed in the first collected stool (analyzed in the ATP and ITT populations). The day by treatment interaction term was considered for inclusion in models if statistically significant, and if included, the difference in efficacy measures in the last inpatient day was reported. Proportional odds models were used for the analysis of categorical endpoints (e.g., stool consistency and diarrhea severity). Upper 95% confidence intervals (CI) and p-values were derived from each model.

The safety population consisted of all subjects that received at least one dose of study drug. All safety analyses were descriptive. Ten subjects enrolled in Part B were matched to the first 10 subjects who completed Part A ATP, to develop a comparative description of the absorption and excretion of the drug in the two groups. The PK population consisted of all subjects who had at least one measurable PK concentration. Plasma and stool drug

concentrations were plotted at each timepoint for matched Part A and Part B subjects together, on linear scale. PK parameters were estimated through a non-compartmental analysis using Phoenix WinNonlin version 8.0 or later (Pharsight Corporation, Cary, NC, USA). The paired t-test was used to assess differences between groups for each PK parameter on days 1 and 5 (C_{min}, C_{max}, and AUC_{0-24}), and reported the geometric mean ratio between groups. We reported the Hodges-Lehmann estimator (pseudomedian) for the difference in each parameter between Part A and Part B subjects. P-values and 95% CI for each of the above tests were calculated.

Due to the exploratory nature of the trial, no adjustments due to multiple testing were made; all statistical tests were performed with a one-sided alpha of 0.05. Statistical analyses were conducted using SAS version 9.3.

To maximize the safety and integrity of the study, an independent data safety monitoring board (DSMB) was involved in regular review of blinded safety data to monitor risks and benefits and to assess any potential safety issues arising during the study. Trial site monitoring of participant safety was carried out by the sponsor medical monitor, an independent local safety monitor, the CRO medical monitor, and overseen by the chief investigator (WVV). This study is registered with ClinicalTrials.gov, number NCT03341767.

1.7 Role of the funding source
The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The first and last authors (PI, WVV) and the funders had full access to all the data in the study, following data lock. The first and last authors were responsible for the decision to submit for publication.

2. Results

2.1 Stool PK profiles
The total observed daily amount of CFZ eliminated in the feces on days 2 and 5 was not significantly different between Part A and B subjects (Supplementary Figure 3). Less than 2% of the cumulative CFZ doses was recovered in stool in both groups over the five days of stool collection.

2.2 Fatal outcomes
Two CFZ-treated subjects developed a fatal sepsis-like syndrome on day 5 of dosing. The first, judged to be unrelated to study drug, developed severe fatigue on the morning of the last dosing day with documented hypotension and was judged to have sepsis, received ceftriaxone and intravenous fluids, but rapidly died despite therapy. The second fatal case occurred in a subject who developed abdominal pains a day after receipt of CFZ, resolved when CFZ was stopped, then recurred when CFZ was restarted. An abdominal ultrasound demonstrated biliary stones, but a surgical consult could not be organized before the subject died of sepsis-like syndrome. The site judged the death to be related to CFZ administration, although the study medical monitors and DSMB judged this fatal SAE to be unrelated. The third CFZ-treated subject that died presented to the hospital with profound hypotension and diarrhea on day 18 after receipt of study drug. The patient did not respond to fluid resuscitation in the emergency suite and expired quickly after arrival. Death was attributed to the effects of chronic diarrhea, AIDS, and delayed presentation. The fatal SAE in the placebo group occurred 47 days after study drug administration, in a subject who had been diagnosed with pulmonary and extrapulmonary TB after randomization with rehydration. The latter two

deaths were judged not to be related to treatment. No autopsies were conducted in any of the deaths so exact causes of death could not be ascribed.

3. References

1. Nachipo P, Hermann D, Quinnan G, Gordon MA, Van Voorhis WC, Iroh Tam PY. Evaluating the safety, tolerability, pharmacokinetics and efficacy of clofazimine in cryptosporidiosis (CRYPTOFAZ): study protocol for a randomized controlled trial. *Trials* 2018; **19**(1): 456.
2. Liu J, Kabir F, Manneh J, et al. Development and assessment of molecular diagnostic tests for 15 enteropathogens causing childhood diarrhoea: a multicentre study. *Lancet Infect Dis* 2014; **14**(8): 716-24.
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5. Glaberman S, Moore JE, Lowery CJ, et al. Three drinking-water-associated cryptosporidiosis outbreaks, Northern Ireland. *Emerg Infect Dis* 2002; **8**(6): 631-3.

Supplementary Table 1. Efficacy of clofazimine compared to placebo in the according to protocol (ATP) and intention-to-treat (ITT) populations

Outcomes	Difference in means	95% upper confidence limit	P-value
Parasitologic			
Change from baseline in log_2 number of cryptosporidium shed in first collected stool (log_2 *Cryptosporidium* per gram), ATP	2·17	3·82	0·984
Change from baseline in log_2 number of cryptosporidium shed in first collected stool (log_2 *Cryptosporidium* per gram), ITT	1·73	3·13	0·977
Change from baseline in total daily cryptosporidium shedding (log_2 *Cryptosporidium*), ATP	1·02	2·50	0·877
Change from baseline in total daily cryptosporidium shedding (log_2 *Cryptosporidium*), ITT	0·16	1·69	0·569
Diarrheal			
Change from baseline in total stool weight at Day 5 (g), ATP	132·05	314·48	0·888
Change from baseline in total stool weight at Day 5 (g), ITT	-45·30	179·88	0·366
Number of diarrheal episodes, ATP	1·92	5·73	0·802
Number of diarrheal episodes, ITT	2·32	5·74	0·871
Characteristics	Odds ratio		
Most severe stool consistency grade, ATP	0·66	10·39	0·401
Most severe stool consistency grade, ITT	1·85	26·26	0·651
Most severe diarrhea grade, ATP	5·33	29·28	0·947
Most severe diarrhea grade, ITT	4·87	23·85	0·950

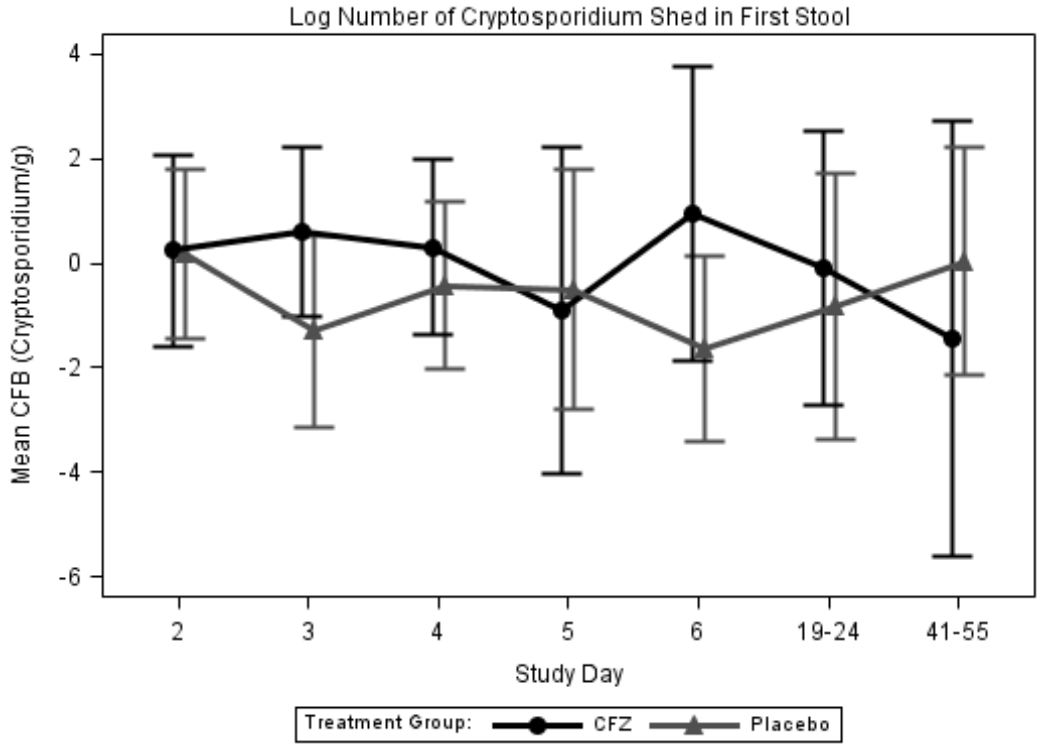
Supplementary Table 2. Total number of unsolicited adverse events

		Part A – CFZ (n=12)	Part A – placebo (n=10)	Part B (n=11)
MedDRA® system organ class	MedDRA® preferred term	No. of events	No. of events	No. of events
Any system organ class	Any preferred term	13	12	3
Blood and lymphatic system disorders	Anemia	0	3	0
Gastrointestinal disorders	Any preferred term	4	0	0
	Abdominal pain	1	0	0
	Anal fissure	1	0	0
	Diarrhea	2	0	0
General disorders and administration site conditions	Pyrexia	0	1	0
Infections and infestations	Any preferred term	4	6	0
	Extrapulmonary tuberculosis	0	1	0
	Gastroenteritis	1	1	0
	Lower respiratory tract infection	1	0	0
	Esophageal candidiasis	0	1	0
	Oral candidiasis	0	1	0
	Pneumonia	0	1	0
	Pulmonary tuberculosis	0	1	0
	Sepsis	1	0	0
	Septic shock	1	0	0
Investigations	Any preferred term	1	0	3
	Alanine aminotransferase increased	0	0	2
	Neutrophil count decreased	0	0	1
	White blood cell count decreased	1	0	0
Metabolism and nutrition disorders	Hypokalemia	1	1	0
Skin and subcutaneous tissue disorders	Decubitus ulcer	1	0	0
Vascular disorders	Any preferred term	2	1	0
	Hypotension	1	1	0
	Hypovolemic shock	1	0	0

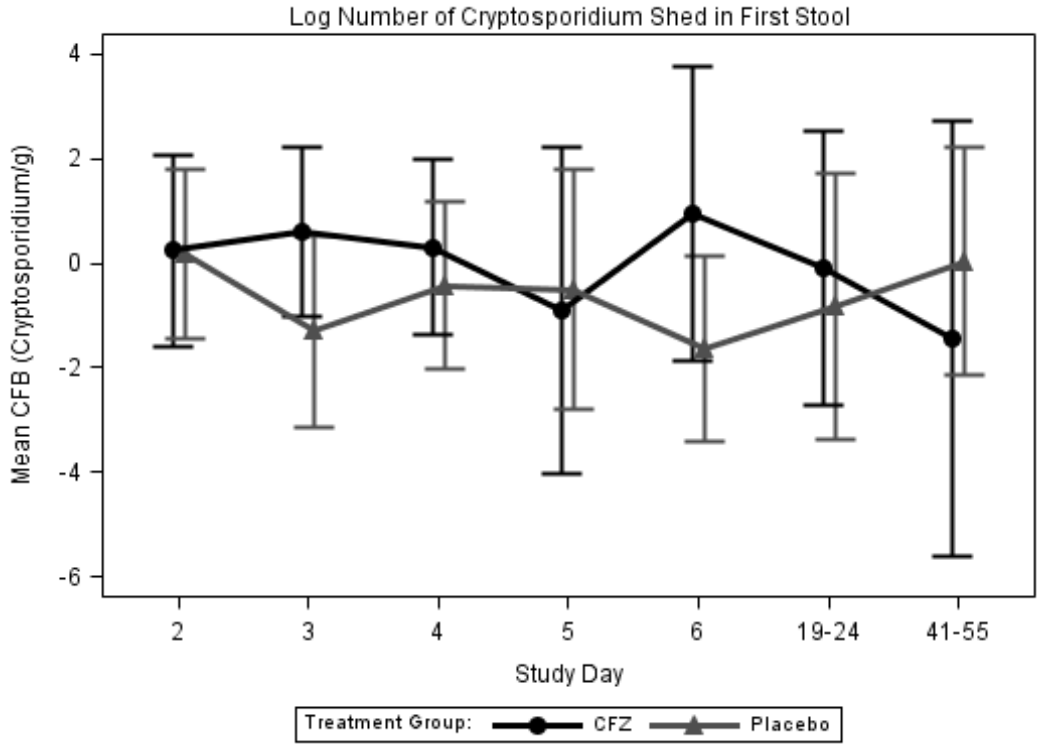
CFZ, clofazimine; MedDRA®, medical dictionary for regulatory activities

Supplementary Figure 1. Treatment response in the intention-to-treat group:

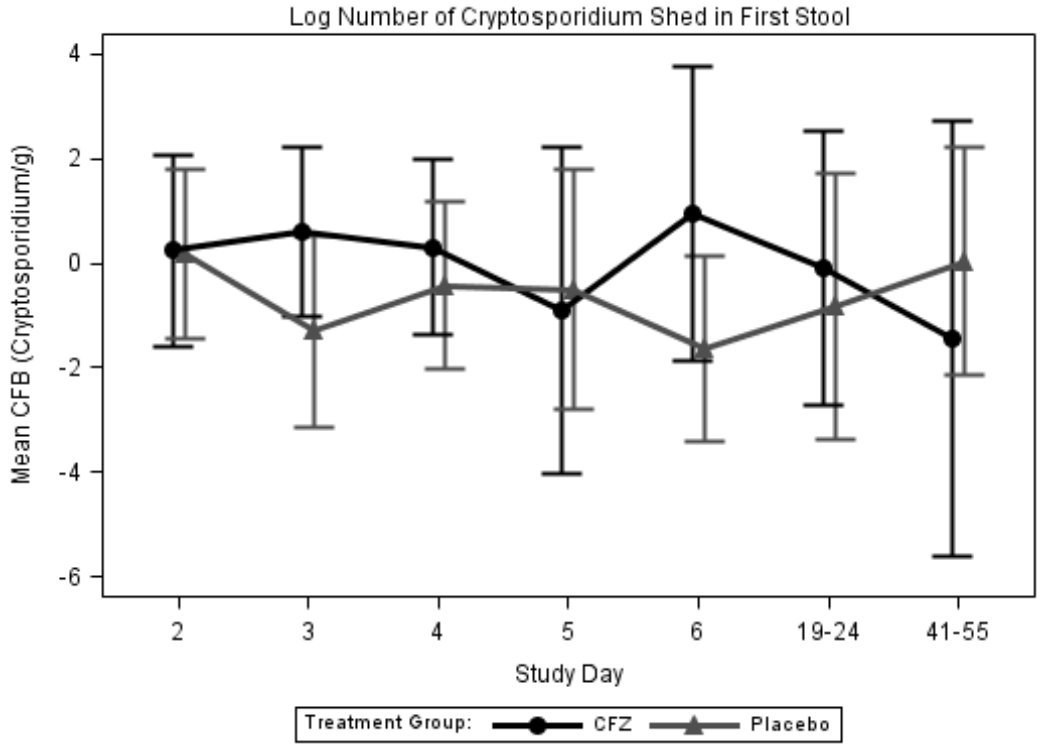
A) Mean change from baseline (CFB) in $\log_2$ number of cryptosporidium shed in first collected stool over time



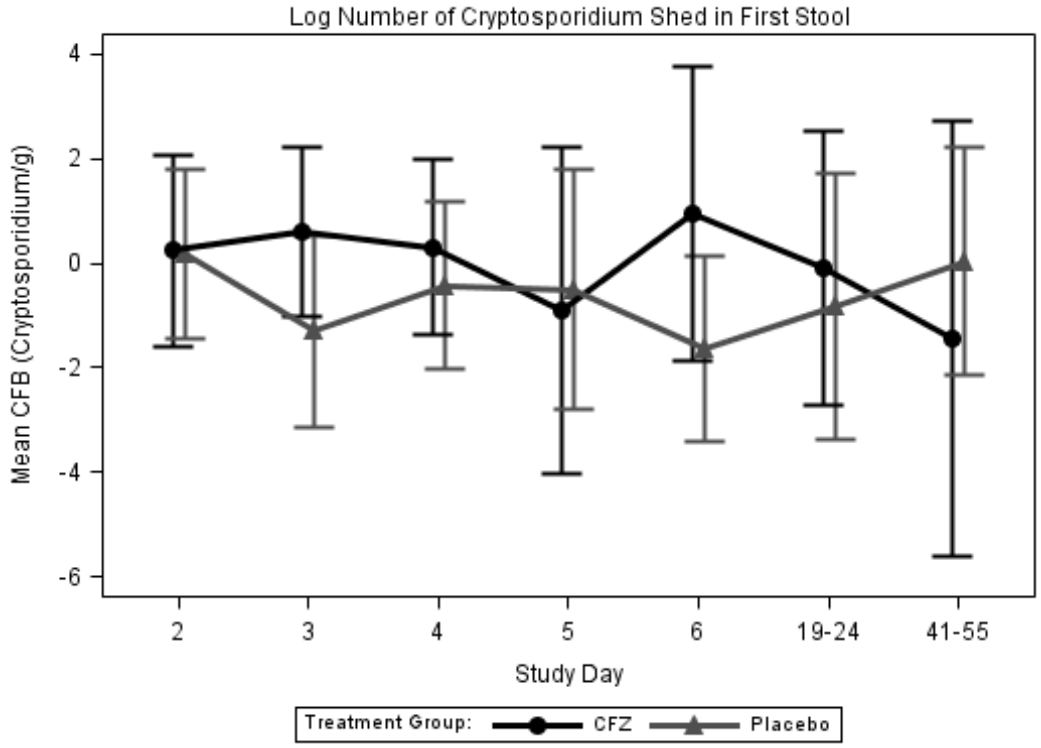
B) Mean CFB in total daily cryptosporidium shedding over time



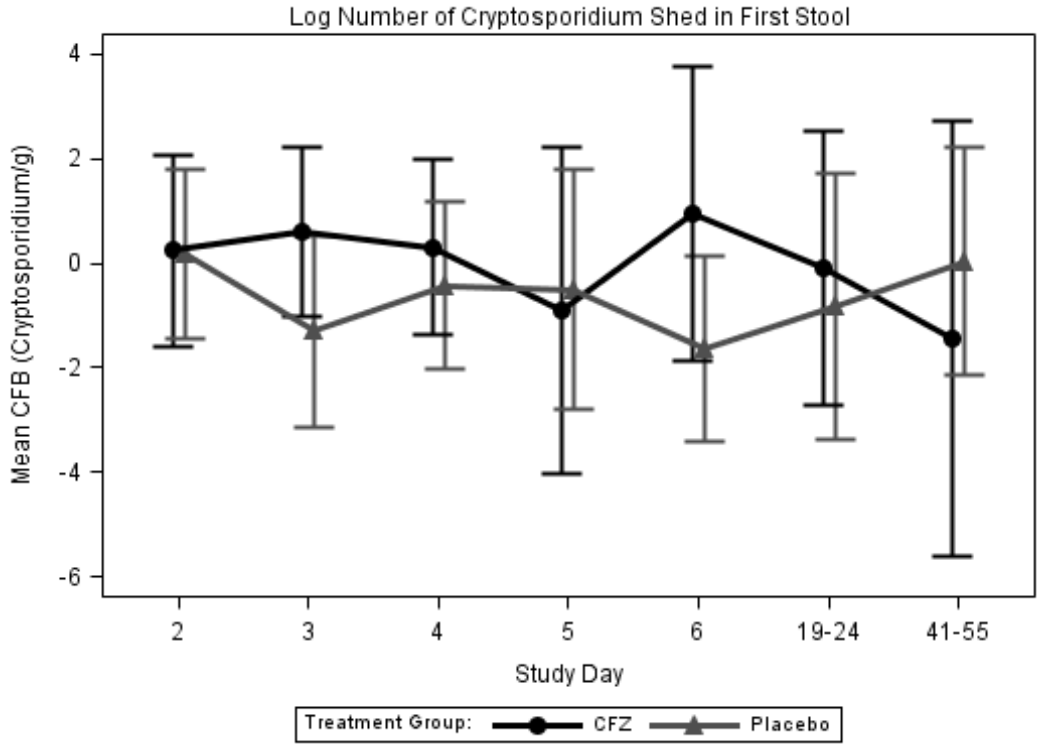
C) Mean total daily cryptosporidium shedding over time



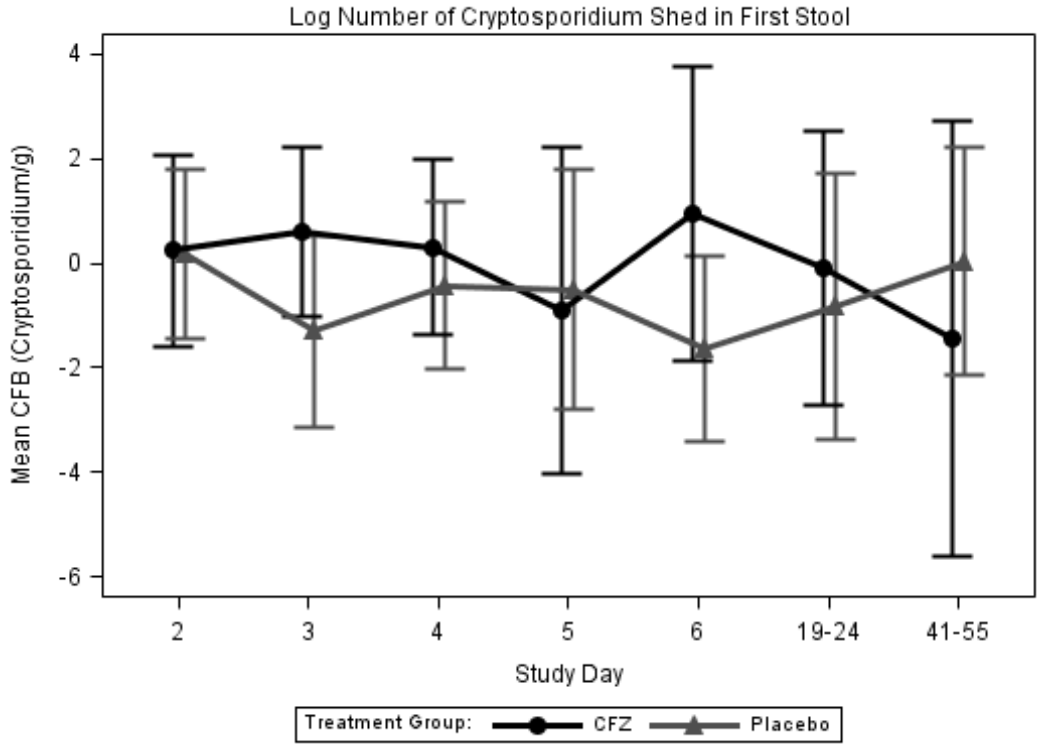
D) Mean CFB in total stool weight over time



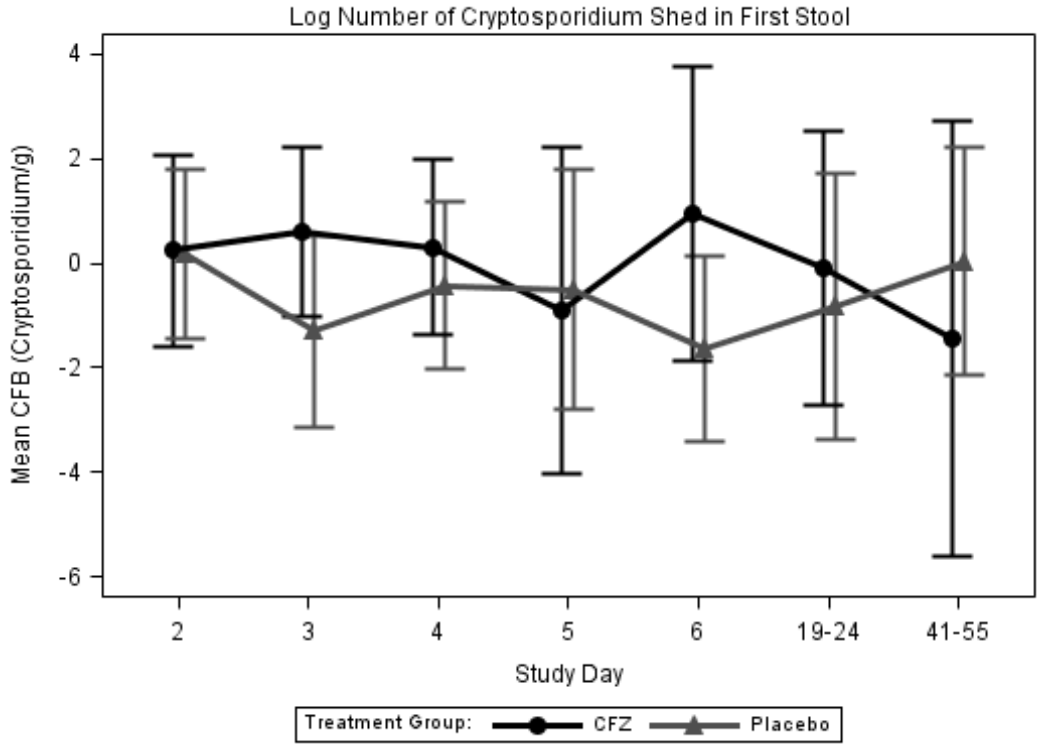
E) Mean number of diarrheal episodes over time



F) Proportion of most severe stool consistency grade by time

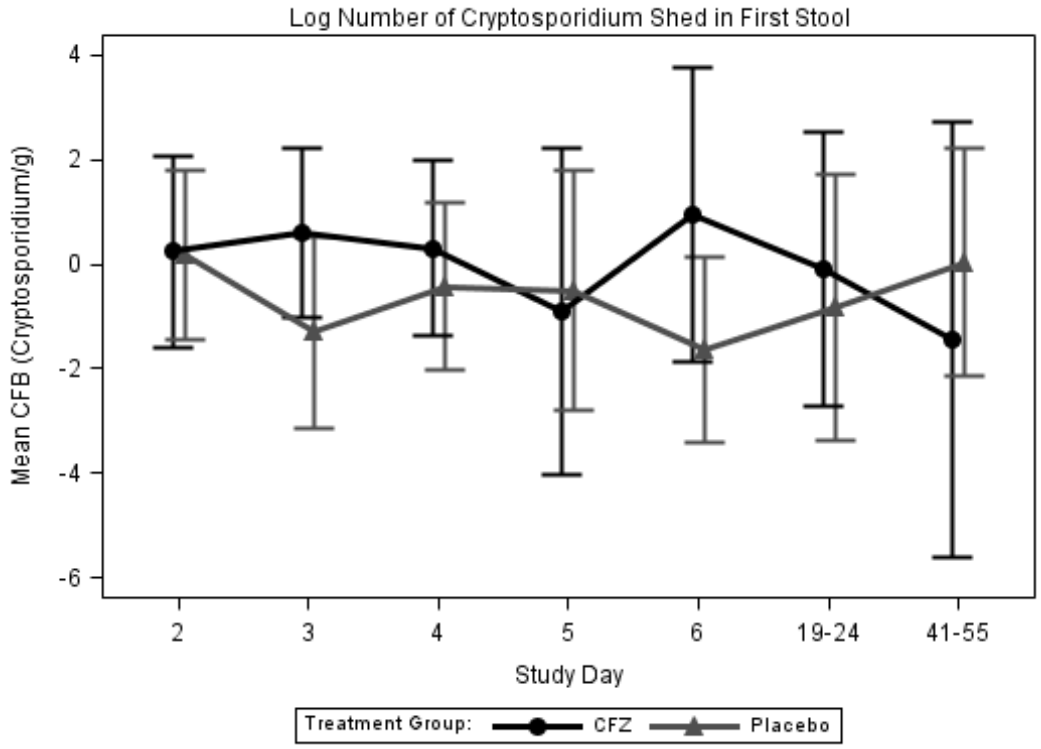


G) Proportion of most severe diarrhea grade by time

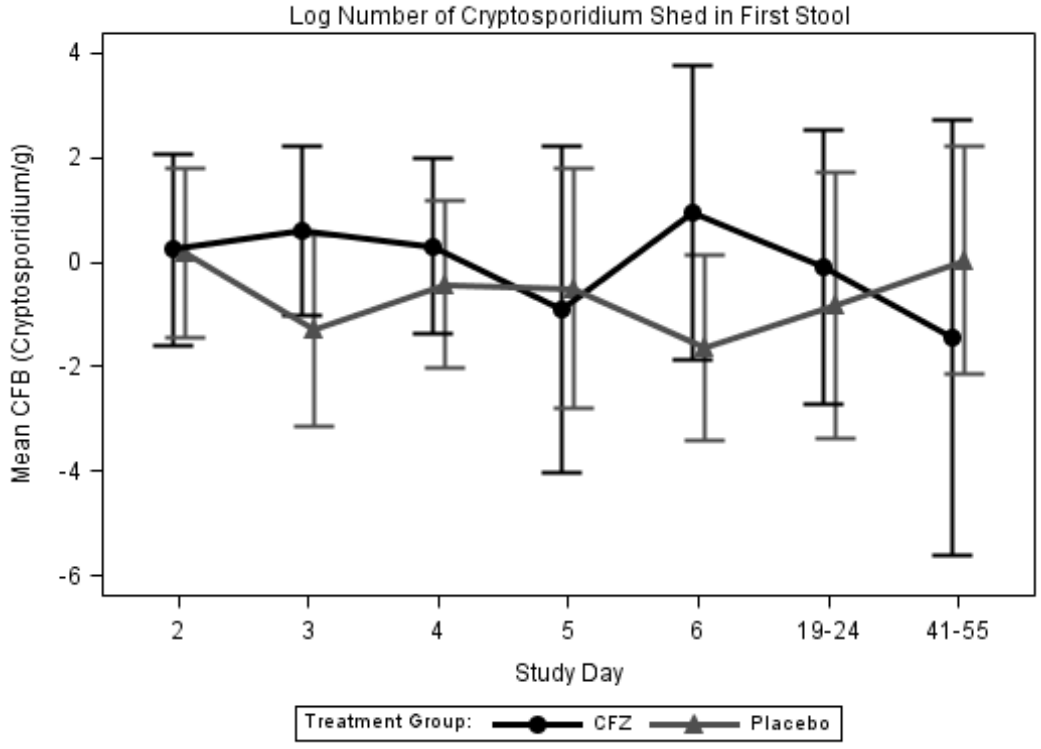


Supplementary Figure 2. Stool cryptosporidium shedding in:

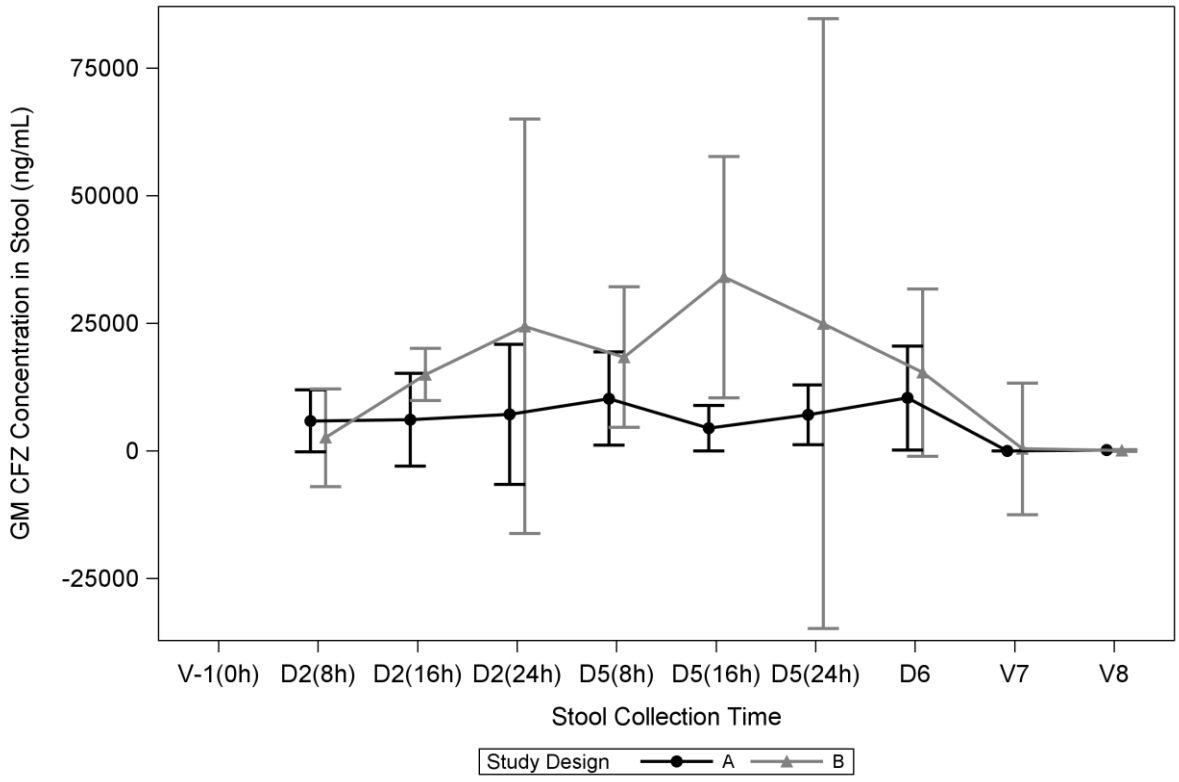
A) First stool of the day



B) Total daily stooling

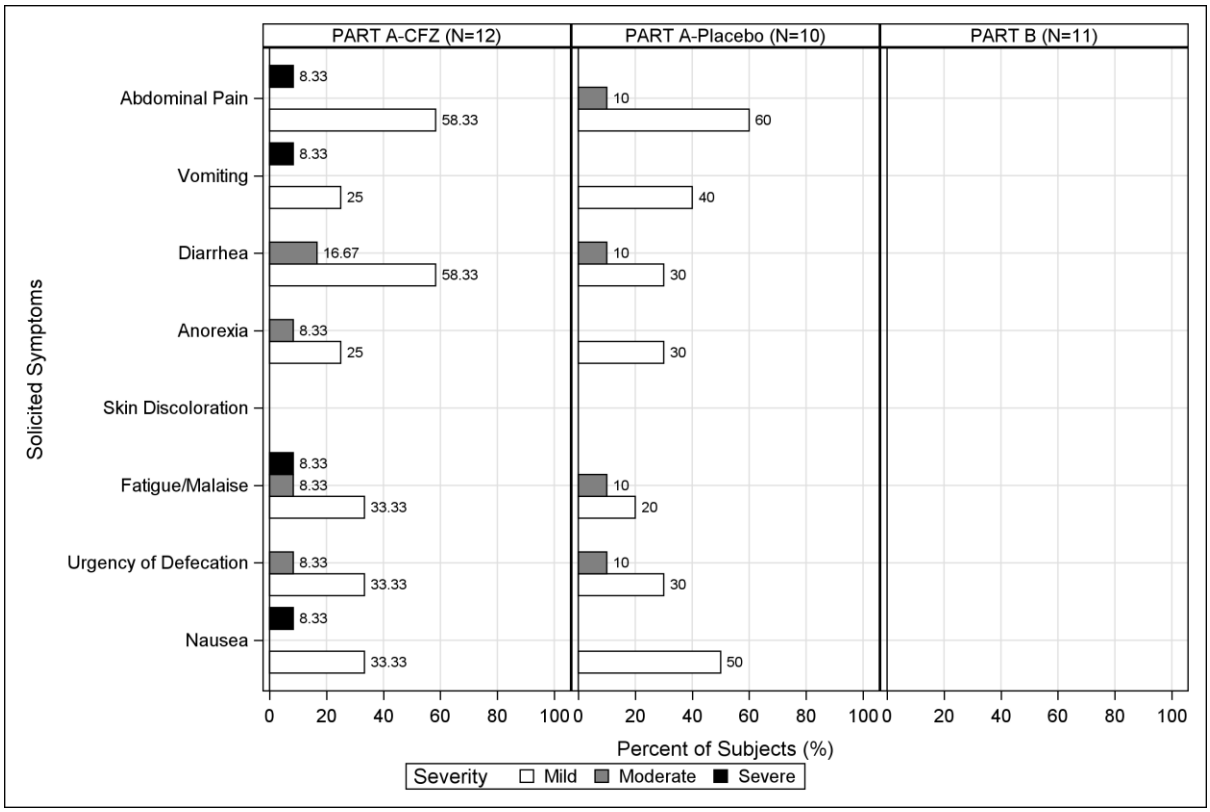


Supplementary Figure 3. Mean amount of CFZ in stool by timepoint



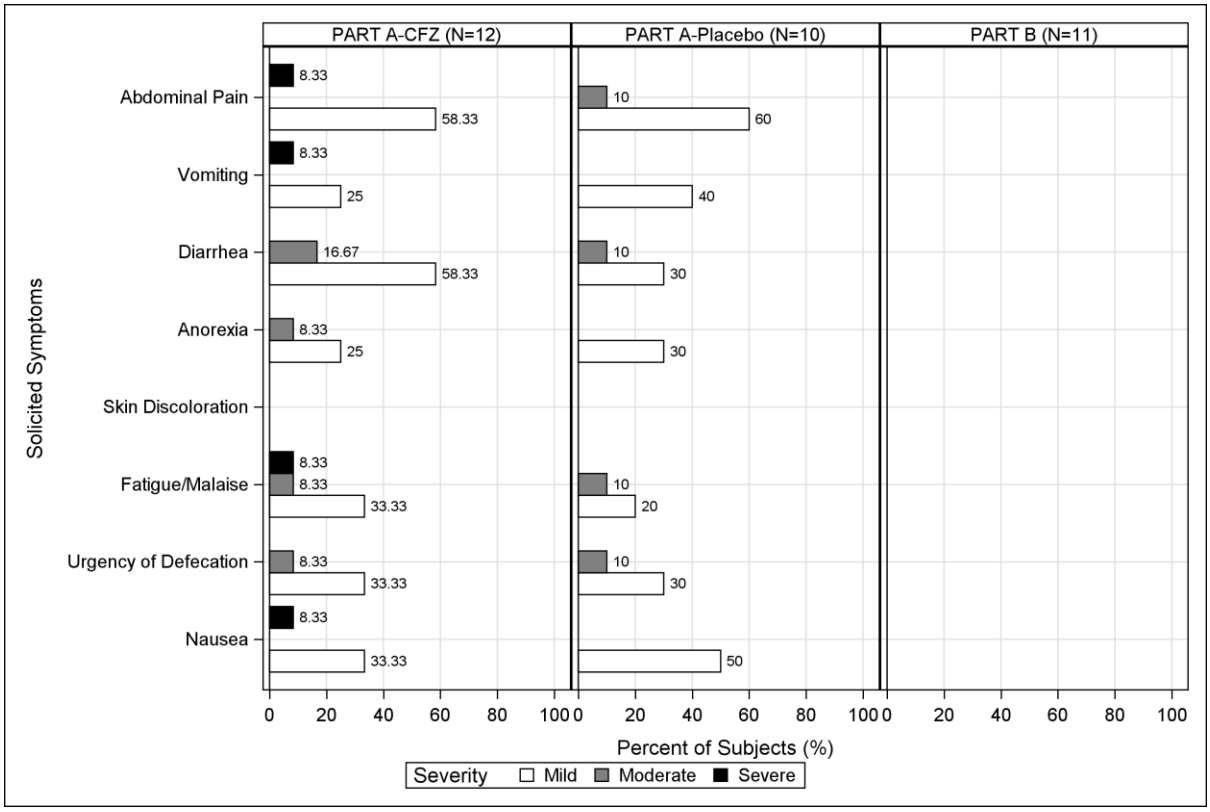
CFZ, clofazimine; D, day; GM, geometric mean; V, study visit

Supplementary Figure 4. Maximum severity of solicited symptoms

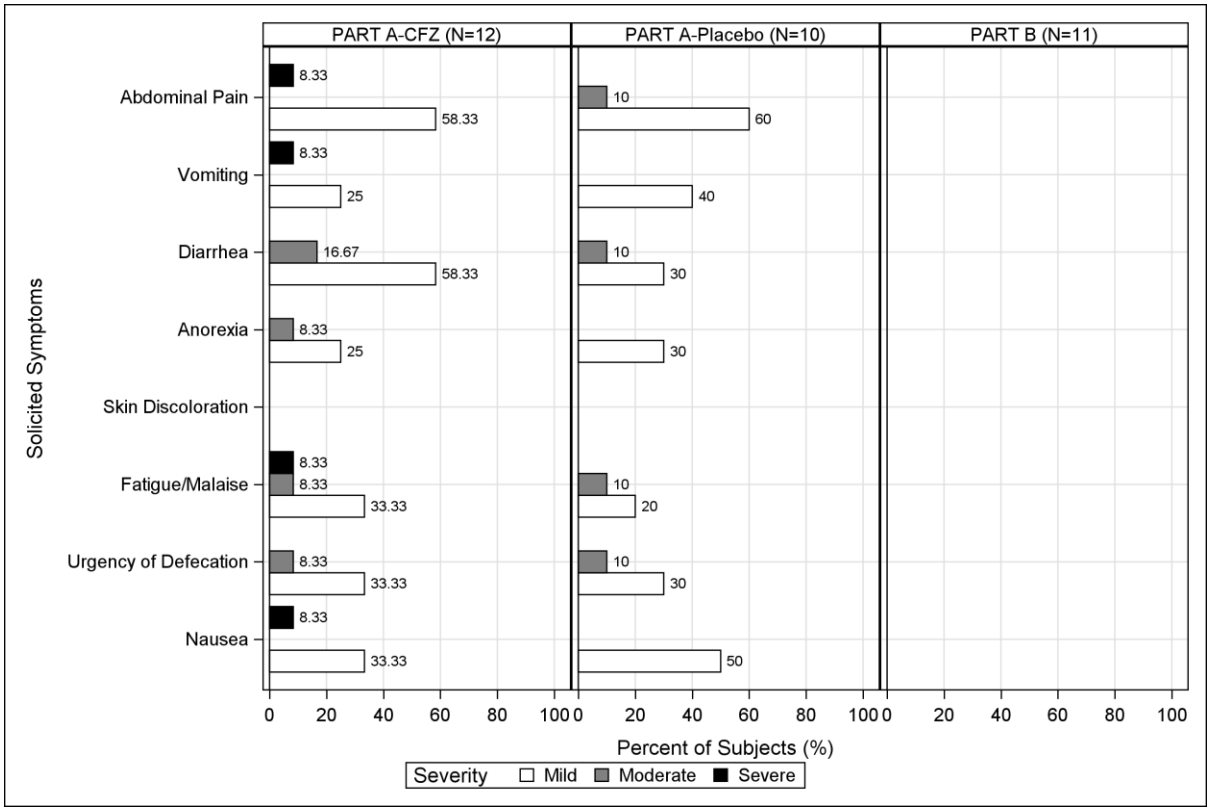


Supplementary Figure 5. Frequency of adverse events by organ class and:

A) Severity



B) Relationship to treatment



Clofazimine for treatment of cryptosporidiosis in HIV-infected adults (CRYPTOFAZ): an experimental medicine, randomized, double-blind, placebo-controlled phase 2a trial

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Brief title: Clofazimine trial for cryptosporidiosis

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Key points

We evaluated clofazimine for treatment of adult HIV subjects with cryptosporidiosis.

Clofazimine was well tolerated, but did not reduce *Cryptosporidium* excretion or diarrhea compared with subjects treated with placebo. This trial forms a blueprint for future cryptosporidiosis therapeutic trials.

Abstract

Background: We evaluated efficacy, pharmacokinetics (PK), and safety of clofazimine (CFZ) in HIV-infected patients with cryptosporidiosis~~, a life-threatening infection without effective treatment for this population~~.

Methods: We performed a randomized, double-blind, placebo-controlled study. Primary outcomes in Part A were reduction in *Cryptosporidium* shedding, safety, and PK. Primary analysis was according to protocol (ATP)~~ with intention-to-treat as secondary analysis~~. Part B of the study compared ~~the~~ CFZ PK ~~of CFZ~~ in matched HIV-infected individuals without cryptosporidiosis~~ (Clinicaltrials.gov #NCT03341767)~~.

Results: Twenty Part A and 10 Part B participants completed the study ATP. Almost all Part A participants had high viral loads and low CD4 counts, consistent with failure of antiretroviral (ARV) therapy. At study entry, the Part A CFZ group had higher *Cryptosporidium* shedding, total stool weight, and more diarrheal episodes compared to the placebo group. Over the inpatient period, compared to those who received placebo, the CFZ group *Cryptosporidium* shedding increased by 2.17 $\log_2$ *Cryptosporidium* per gram stool (95% upper confidence limit: 3.82), total stool weight decreased by 45.3 g (p=0.37), and number of diarrheal episodes increased by 2.32 (p=0.87). The most frequent solicited adverse effects were diarrhea~~ (9/12, 75%)~~, abdominal pain~~ (8/12, 67%)~~, and malaise~~ (6/12, 50%)~~. Three CFZ and 1 placebo subjects died during the study. Plasma levels of CFZ in participants with cryptosporidiosis were 2-fold lower than Part B controls. ~~Part A subjects continued shedding *Cryptosporidium* up to 60 days after screening.~~

Conclusion: Our findings do not support the efficacy of CFZ for the treatment of cryptosporidiosis in a severely immunocompromised HIV population. However, this trial demonstrates a pathway to assess the therapeutic potential of drugs for cryptosporidiosis treatment. Screening persons with HIV for diarrhea, and especially *Cryptosporidium* infection, may identify those failing ARV therapy.

250 words

Keywords: Cryptosporidium, diarrhea, HIV, therapeutic, trial

Introduction

Cryptosporidium infection and diarrhea (cryptosporidiosis) is a life-threatening infection in persons with HIV and also in young children in the developing world [1]. In children, cryptosporidiosis causes severe diarrhea [2], malabsorption and intestinal injury [3], excess mortality [2, 4], stunting and is associated with malnutrition [5]. ~~To date, only nitazoxanide is licensed for treatment of cryptosporidiosis, but it has not shown any benefits as a treatment for HIV-infected and immunocompromised patients with cryptosporidiosis compared to placebo [6-8]. Therefore, t~~There is a huge unmet need for *Cryptosporidium* drugs [6]: only nitazoxanide is licensed for treatment of cryptosporidiosis, but it has not shown any benefits as a treatment for HIV-infected and immunocompromised patients with cryptosporidiosis compared to placebo [7-9]. ~~.~~

Clofazimine (CFZ), used for treatment of leprosy for more than 50 years, and currently part of treatment for multi-drug resistant TB, has recently been described as effective against *Cryptosporidium in vitro* ~~, and was able to eliminate *C. parvum* in a mouse model~~ [10]. ~~CFZ has been in use for treatment of leprosy for more than 50 years, and is also used as part of a WHO regimen to treat multi-drug resistant *Mycobacterium tuberculosis*.~~ The efficacy and pharmacokinetics (PK) of CFZ in HIV-infected patients with cryptosporidiosis are not known.

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We developed an experimental medicine study design to evaluate the safety, tolerability, PK and efficacy of CFZ in HIV-infected adults with cryptosporidiosis.

Methods

Study design and participants

The study was a single center, randomized, double-blind, placebo-controlled Phase 2a two-part study at Queen Elizabeth Central Hospital in Blantyre, Malawi. Participants were eligible for Part A if they were HIV-infected, aged 18-65 years, weight over 35·4 kg, on antiretrovirals (ARV) for at least 1 month, and with diarrhea duration of minimum 14 days. Participants for Part B were HIV-infected without diarrhea or *Cryptosporidium*, and met none of the exclusion criteria. Full ~~inclusion and exclusion~~ criteria ~~for Part A and B participants~~ are listed in the Supplementary Appendix. The study protocol ~~and relevant supporting materials were~~was approved by the relevant regulatory and ethics committees before study initiation [11]. Participants provided written informed consent.

Study treatment and procedures

Part A participants were randomized 1:1 to receive either five days of oral CFZ or placebo, respectively (Figure 1). The dosage of CFZ administered was the maximum given in clinical practice, 100 mg three times daily if ≥50 kg or 50 mg three times daily for subjects <50 kg [12]. Participants for Part B were matched 1:1 to the first ten Part A subjects based on age (±5 years), gender, and weight (≥ or <50 kg; Supplementary Appendix).

We used a rapid diagnostic test (RDT) for *Cryptosporidium* screening (prototype immunochromatographic test strip for detecting *Cryptosporidium*, TechLabs Inc., Blacksburg, VA, USA) and an ELISA stool test (CRYPTOSPORIDIUM II™, TechLabs Inc.) for assessing *Cryptosporidium* shedding in serial stools during the trial. All *Cryptosporidium* shedding was confirmed and measured by qPCR, with a positive result being a cycle threshold (Ct) <35. The first collected stool of the day was obtained throughout the dosing and follow-up periods, for testing of the *Cryptosporidium* ELISA signal, as well as for measurement of *Cryptosporidium* shedding by qPCR. In addition, all stools were collected and pooled in 8-hour intervals during the inpatient phase of the study, Days -1 to 5 of dosing. Thus, total *Cryptosporidium* stool excretion was measured by qPCR during this time.

Stool enteropathogens present at baseline in addition to *Cryptosporidium* were detected using qPCR in a TaqMan Array Card (TAC, Thermo Fisher, Waltham, MA, USA) using a custom design developed at the Houpt Laboratory (Charlottesville, VA, USA; Supplementary Appendix) [13]. Measurements of anti-retroviral (ARV) levels in plasma and alteration after administration of CFZ were evaluated in the Van Voorhis/Arnold Laboratories (Seattle, WA, USA). Measurement of CFZ concentration in plasma and stool were performed at Q_2 Solutions (Ithaca, NY, USA).

After the 5-day inpatient study drug dosing, with daily clinical examination and laboratory sampling, all participants entered a ~~two~~ 2 month follow-up period that included a visit 19-24 days post last dose, and a final visit 41-55 days post last dose. During each visit and with weekly phone calls, participants were monitored for safety and symptoms. Safety labs were repeated if

there were any abnormalities previously. If participants could not be reached by phone, home visits were made.

Outcomes

There were two primary endpoints for Part A: the first was efficacy, assessed as reduction in the (log) number of *Cryptosporidium* shed in the first collected stool of each study dosing day of CFZ vs. placebo recipients in subjects treated according to protocol (ATP). The second primary endpoint was safety, ~~and consisted of~~including frequency and severity of solicited and unsolicited adverse events (AEs), ~~including~~ serious adverse events (SAEs), adverse events of special interest and suspected, unexpected serious adverse reactions. Part B had two primary endpoints (CFZ in plasma, and total daily amount of CFZ eliminated in stool) to meet a single primary PK objective. Secondary endpoints were the reduction in the (log_2) number of *Cryptosporidium* shed in stool compared to controls in the intention-to-treat (ITT) population, reduction in total daily *Cryptosporidium* shedding in those treated ATP, and as compared to controls in the ITT population, and reduction in severity of diarrhea over the study dosing period compared to controls.

An independent data safety monitoring board (DSMB) was involved in regular review of blinded safety data to monitor risks and benefits and to assess any potential safety issues arising during the study. Trial site monitoring of participant safety was carried out by the sponsor medical monitor, an independent local safety monitor, the contract research organization medical monitor, and overseen by the chief investigator (WVV). This study is registered with ClinicalTrials.gov, number NCT03341767.

184

185 Statistical analyses

186 As the Phase 2a study was exploratory, we initially planned an interim analysis after 20 subjects
187 were randomized and treated ATP; this sample size was predicted to detect a therapeutic
188 difference based on animal data from molecular endpoints. Due to slow enrollment, it was
189 decided to convert the interim analysis to a final analysis (Supplementary Appendix).

190

191 The primary ATP analysis was performed using the randomized population who received at least
192 80% of scheduled doses, completed daily assessments of fecal shedding, and had no major
193 protocol deviations. When missing data for the primary endpoint (log number of
194 *Cryptosporidium* shed per gram stool) was not attributable to non-detectable *Cryptosporidium*
195 (i.e. no stooling), multiple imputation was utilized (Supplementary Appendix).

196

197 The safety population consisted of all subjects that received at least one dose of study drug. The
198 PK population consisted of all subjects who had at least one measurable PK concentration
199 (Supplementary Appendix).

200

201 Due to the exploratory nature of the trial, no adjustments due to multiple testing were made; all
202 statistical tests were performed with a one-sided alpha of 0.05. Statistical analyses were
203 conducted using SAS version 9.3.

204

205 Results

~~In the Blantyre District of Malawi, b~~Between 18 December 2017 and 14 February 2019, 5,790 adults were approached to assess eligibility. For randomization to CFZ vs. placebo (Part A), 494 were prescreened for *Cryptospo~~di~~ridium* presence in stool via RDT and qPCR, 67 participants were *Cryptosporidium* PCR-positive in stool and screened, and 22 were randomized (12 to CFZ and 10 to placebo, ITT group; Figure 1). Twenty subjects completed inpatient dosing ATP. There was one voluntary withdrawal (CFZ group) during the outpatient phase. There was no loss to follow-up.

The RDT and ELISA stool test had low sensitivity (41% for both) to identify participants and follow the presence/absence of *Cryptosporidium* over time, compared with qPCR. The *Cryptosporidium* spp. identified were *C. parvum* (11/22, 50%), *C. meleagridis* (4/22, 18%), *C. hominis (*3/22, 14%), *C. viatorum* (1/22, 5%) and 3 unknowns. Coinfection of stool with multiple diarrhea enteropathogens was common, with a median of 4 co-pathogens (excluding *Cryptosporidium*) per subject (range 1-8). The most frequently identified co-pathogen was enteroaggregative *E. coli* (64%), followed by *Shigella* toxin-positive enterotoxigenic *E. coli* (41%) and *Shigella*/enteroinvasive *E. coli* (23%). The baseline characteristics of participants are listed in Table 1. Despite randomization, compared to the placebo group the CFZ group had by chance: more males (67% vs. 20%), lower body mass index (16.3±1.7 vs. 18.0±3.1 kg/m^2), increased diarrhea output total stool weight (320.3±214.6 vs. 245.8±299.4 g), more pathogens detected at ~~higher quantities~~a diarrheagenic amount per Global Enteric Multicenter Study (GEMS) criteria (67% vs. 30%) [14], more advanced HIV immunosuppression (CD4 counts 25.3±24.4 vs. 170.4±321.7 cells/μL), and higher prevalence of *C. parvum* detected (58% vs. 40%).

Findings were similar for both ATP and ITT populations (Supplementary Table 1), and the ATP efficacy results are reported here. Stool *Cryptosporidium* excretion was persistent among Part A subjects throughout observation (Supplementary Figures 1 and 2), even at 41-55 days after the last dose. There was no significant difference in *Cryptosporidium* shedding in the CFZ group compared to placebo (Figures 2A-B). There was a trend towards increased change-from-baseline in *Cryptosporidium* shedding in the first stool of the day in the CFZ-treated group vs. placebo, with a difference in means of 2.17 $\log_2$ *Cryptosporidium* per gram ([95% upper confidence limit (CL): 3.82]), and in total *Cryptosporidium* shedding with a difference of means of 1.02 $\log_2$ *Cryptosporidium* ([95% upper CL: 2.50]); the opposite result expected if CFZ was efficacious. There was no significant change in diarrhea in the CFZ group compared to placebo, whether measured by total stool weight change-from-baseline, number of diarrheal episodes, stool consistency grade, or severity diarrhea grade (Figures 2C-F).

For the PK of CFZ in HIV-infected subjects without diarrhea or *Cryptosporidium* (Part B), 92 were prescreened, 18 were screened, and 11 received CFZ, with one voluntary withdrawal during the inpatient phase. Part A subjects had about 2-fold less plasma exposure of CFZ than Part B subjects on day 5 (ratio $AUC_{0\text{-}24}$: 0.607), and on day 1 of the inpatient dosing (ratio $AUC_{0\text{-}24}$: 0.478; Table 2, Figure 3; stool PK profiles are listed in Supplementary Appendix and Supplementary Figure 3).

For safety, solicited AEs (Table 3) - expected in persons with diarrhea - were experienced by all subjects in both CFZ and placebo groups. There were higher numbers of solicited AEs

experienced in the CFZ group for diarrhea (9 (75%) vs. 4 (40%) in placebo), abdominal pain (8 (67%) vs. 7 (70%) in placebo), and malaise (6 (50%) vs. 3 (30%) in placebo), and more severe solicited AEs in the CFZ group (2 (17%)) than the placebo group (0 (0%); Supplementary Figures 4 and 5). -No Part B subject experienced any solicited AE. The number of unsolicited AEs (Supplementary Table 2) was highest in the CFZ group (13 vs. 12 in placebo and 3 in Part B); the number of subjects who experienced AEs with fatal outcome was also higher in the CFZ group (3 (25%) vs. 1 (10%) in placebo and none in Part B~~; Supplementary Appendix~~). None of the fatalities were judged by the study medical monitors and DSMB to be CFZ-related (Supplementary Appendix).

Discussion

This is the first randomized, double-blind, placebo-controlled Phase 2a trial to evaluate CFZ for treatment of cryptosporidiosis in HIV-infected adults. The trial demonstrated that CFZ had no significant impact on *Cryptosporidium* shedding of the parasite, or on diarrheal episodes, stool weight, and consistency, compared to placebo. Evaluation of *Cryptosporidium* shedding in the first stool of the day provided similar data to total daily *Cryptosporidium* shedding. The drug is generally well-tolerated. Four patients died, three of whom received CFZ and the fourth placebo. This rate of death was consistent with our a priori estimates and each case was reviewed by the independent DSMB. CFZ achieved 2-fold less plasma exposure among Part A subjects with diarrhea vs. Part B subjects without diarrhea.

The trial did show that HIV-infected adults with ≥3 days of diarrhea consistently excreted *Cryptosporidium* in their stools, even when assayed up to 60 days after enrollment. This

demonstrates that this population would be appropriate to study the antiparasitic benefit of anti-*Cryptosporidium* drugs that do not depend on the immune response.

The trial did not show a reduction in *Cryptosporidium* excretion in this population treated with CFZ vs. placebo. This was the case whether one compared the *Cryptosporidium* excretion by qPCR as determined by the concentration in the first stool of the day, or by determining the total *Cryptosporidium* excreted per day. In fact, there was a non-significant trend towards slightly increased *Cryptosporidium* shedding in the CFZ group vs. the placebo, which was most evident at day 2 of study drug dosing. The trend towards increased shedding may reflect the more ill status of the CFZ subjects at baseline, as documented in their enrollment labs and health status. With a median HIV CD4 count of 23.5 cells/mm^3 (IQR 11.75, 43.75) and viral load of 168,097.5 copies/mL (IQR 94,044, 643,812.3), the mortality rate of 18% in the trial likely reflects advanced disease in our Part A cohort as a whole.

Within our cohort, compared to placebo, the CFZ group had more deaths, SAEs, and severe solicited AEs. All subjects with cryptosporidiosis reported the solicited AEs expected with CFZ, such as diarrhea, abdominal pain, malaise and nausea. However, these solicited AEs were present at baseline in Part A subjects, as might be expected in this population with cryptosporidiosis, and ~~solicited AEs~~ were universal in both treatment groups. There tended to be less solicited AEs over time, which correlated with less severity in diarrhea during the hospital phase, and the severity of AEs tended to decrease over time. None of the Part B ~~HIV-infected~~ subjects ~~without cryptosporidiosis~~ exposed to the same dose of CFZ reported solicited AEs, and only 3 Part B subjects reported unsolicited AEs, and these were generally mild.

A previous clinical trial for cryptosporidiosis treatment identified multiple safety concerns related to the health status of participants. This Phase 1-2 trial of miltefosine to treat HIV-related cryptosporidiosis in Zambian adults with chronic diarrhea was terminated early due to high mortality, lack of efficacy and development of SAEs that were attributed to the extreme metabolic abnormalities already present in patients enrolled in the trial [15]. In our trial, subjects with cryptosporidiosis also presented with electrolyte abnormalities, most commonly hypokalemia that required correction, and ~~for~~ some ~~subjects~~ required corrective treatment ~~continued~~ through the trial. In addition, there was also a very high incidence of active TB in the HIV-infected screening population. Screening by chest x-ray was inadequate likely because dehydrated subjects often do not have an infiltrate until rehydrated. Screening of sputum by GeneXpert or gram stain also was inadequate due to inability of dehydrated subjects to produce sputum. ~~Urine LAM screening was instituted based on findings from other studies describing urine LAM as a predictor of disseminated TB and mortality in HIV-infected adults with low CD4 counts [16].~~ All deaths in our study were reported prior to instituting urine LAM screening at baseline. Once urine LAM screening was instituted [16], ~~Notably,~~ 43% of our otherwise eligible subjects subsequently tested positive by urine LAM and were excluded.

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Part A participants were extremely immunosuppressed. Most had CD4 counts <25 cells/μL and high HIV viral loads. Plasma levels of HIV medicines were detected at similar levels to Part B subjects (unpublished data), suggesting that these Part A subjects were compliant with first-line ARV therapy and that ARV resistance might be driving HIV treatment failure. Therefore,

screening for diarrhea in this population, and especially for *Cryptosporidium*, delineated those more at risk for TB and ARV failure.

The predominant *Cryptosporidium* species was *C. parvum* subtype family IIc anthroponotic (10/11, 91% of those with *C. parvum*). This was unexpected, given that the majority of *Cryptosporidium* species identified in the pediatric ~~Global Enteric Multicenter Study (~~GEMS~~)~~ and adult studies were *C. hominis* [17-20]. However, a high prevalence of *C. parvum* has been noted in HIV/AIDS patients in Ethiopia, where 92/140 (66%) of HIV/AIDS patients were positive by PCR-RFLP [21]. As *C. parvum* has been ~~observed to be~~ associated with prolonged diarrhea in HIV-positive persons more frequently than *C. hominis* [17] the trial inclusion criteria may have selected for this species.

Multiple copathogens were observed in stool, which may have contributed to the diarrhea [3], but ~~*Cryptosporidium* may have driven the diarrhea in at least 15 of 22 subjects in this trial.~~patients with symptomatic diarrhea were routinely treated with ciprofloxacin as standard of care. ~~In these 15 subjects,~~ *Cryptosporidium* may have driven the diarrhea in at least 15 of 22 subjects in this trial, as it ~~*Cryptosporidium*~~ was the pathogen with the lowest C_t value, and may have been the pathogen in the greatest quantity shed in stool. After applying GEMS cutoffs, which ~~are based on~~use C_t counts to determine clinically relevant diarrhea [14], only 7 *Cryptosporidium* samples met diarrheagenic cutoffs, and only 11 samples met diarrheagenic pathogen criteria. ~~Given that~~As GEMS data were based on children, the lack of correlation between C_t value and clinical diarrhea likely reflects the differences seen in an adult population with severe HIV immunosuppression with prolonged diarrhea.

Our PK data suggests that diarrhea and/or *Cryptosporidium* infection negatively impacts CFZ plasma exposure. Since efficacy is likely driven by CFZ levels in the parasite, which may not be well-exposed to intraluminal CFZ as it is located in a vacuole under the epithelial plasma membrane, and faces in towards the gut lumen [22], plasma levels may not reflect efficacy as it would for systemic infections. The fact that serum CFZ levels in persons with well-suppressed HIV were twice as high suggests that in the setting of *Cryptosporidium* infection, the drug was not well absorbed. We propose that lower levels of CFZ likely exist in the epithelium layer in the Part A subjects, as passage through the gastrointestinal epithelium is required for access to the plasma. These lower levels may have contributed to the failure of efficacy against *Cryptosporidium*. However, we used the maximum dosage of CFZ that is accepted as safe in this trial [12], therefore increasing the dosage to improve efficacy may not be feasible. An intravenous form of clofazimine, described in the past [23], may have provided better systemic delivery of the drug; however, this was not a formulation available at the time of the trial.

One of the limitations of the study was the small sample size. This led to slightly uneven randomization (12 vs. 10) based on block size. Also, imbalances in the baseline characteristics were noted in the Part A subjects CFZ vs. placebo groups, with the CFZ group being more ill at baseline. One possible confounder was the presence of multiple co-pathogens in the stool, which could have influenced diarrhea resolution.

~~This trial provides important information for testing the efficacy of anti-*Cryptosporidium* therapeutics. There is a huge unmet need for *Cryptosporidium* drugs for young children and~~

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~~immunocompromised individuals [9]. Despite a growing interest in moving promising pre-clinical drugs into clinical trials there was, until this trial, few placebo-controlled trials in adults [24-26] and limited data on how to test the drugs in Phase 2a. This trial shows that HIV-infected adults with *Cryptosporidium* infection excrete Cryptosporidium consistently and thus the effects of treatment on *Cryptosporidium* excretion would be a feasible way to monitor for efficacy in *Cryptosporidium* therapy.~~

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For the conduct of future human experimental trials of cryptosporidiosis in this population, this study suggests that: 1) the screening population should be evaluated for TB detection, through urine LAM, and for electrolyte disturbances, particularly hypokalemia; 2) use of stool RDT in screening and ELISA tests on serial stools is not as sensitive as qPCR, and that we need only use qPCR to enroll and follow participants for *Cryptosporidium* excretion over time; 3) following serial *Cryptosporidium* shedding by qPCR of the first stool of the day, rather than total stool collection, is probably sufficient to assess efficacy; 4) given the ill status of enrolled subjects, an inpatient trial is merited and AEs and deaths may complicate safety evaluation of new study drugs; and 5) future trials would need to be multisite given the slow recruitment rate. There was, until this trial, few placebo-controlled trials in adults [24-26] and limited data on how to test the drugs in Phase 2a. This trial shows that HIV-infected adults with cryptosporidiosis excrete the parasite consistently and thus the effects of treatment on excretion would be a feasible way to monitor for efficacy in *Cryptosporidium* therapy.

In conclusion, ~~CFZ was not effective in this trial at reducing *Cryptosporidium* excretion nor in resolving diarrhea in HIV-infected subjects with cryptosporidiosis. The decreased plasma exposure of CFZ in the Part A vs. Part B subjects may have influenced the efficacy of CFZ on *Cryptosporidium*; however, the highest recommended dose of CFZ was used for this trial, and further studies on safety and perhaps CFZ formulation would be necessary to increase the dose and exposure. T~~this is the first controlled clinical trial to assess the safety, efficacy, and PK of CFZ for treatment of cryptosporidiosis. ~~, and a~~Although CFZ does not show promise as a novel therapeutic for *Cryptosporidium* infection, future human studies can use an approach based on lessons learned in this trial to assess the therapeutic potential of drugs for treatment of cryptosporidiosis.

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Table 1. Baseline characteristics of participants

Table 2. Comparison of pharmacokinetic parameters in Part A and B subjects

Table 3. Summary of adverse events

Figure legends

Figure 1. Part A trial profile

ATP, according to protocol; ITT, intention to treat

[a]Subject died after completing visit.

[b]One subject withdrew during inpatient phase but provided final blood draw.

Figure 2. Treatment response in the according to protocol group:

A) Mean change from baseline (CFB) in log number of cryptosporidium shed in first collected stool over time

B) Mean CFB in total daily cryptosporidium shedding over time

C) Mean CFB in total stool weight over time

D) Mean number of diarrheal episodes over time

E) Proportion of most severe stool consistency grade by time

F) Proportion of most severe diarrhea grade by time

Figure 3. Mean plasma concentration of CFZ in plasma by time

Supplementary Appendix

Supplementary Methods

Supplementary Results

Supplementary Table 1. Efficacy of clofazimine compared to placebo in the according to protocol (ATP) and intention-to-treat (ITT) populations

Supplementary Table 2. Total number of unsolicited adverse events

Supplementary Figure 1. Treatment response in the ITT group:

A) Mean change from baseline (CFB) in $\log_2$ number of cryptosporidium shed in first collected stool over time

B) Mean CFB in total daily cryptosporidium shedding over time

C) Mean total daily cryptosporidium shedding over time

D) Mean CFB in total stool weight over time

E) Mean number of diarrheal episodes over time

F) Proportion of most severe stool consistency grade by time

G) Proportion of most severe diarrhea grade by time

Supplementary Figure 2. Stool cryptosporidium shedding in: A) First stool of the day B) Total daily stooling

Supplementary Figure 3. Mean amount of CFZ in stool by timepoint

Supplementary Figure 4. Maximum severity of solicited symptoms

Supplementary Figure 5. Frequency of adverse events by organ class and: A) Severity B) Relationship to treatment

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Declaration of interests

PI, KJ, ML, CM and WVV have received grants from Bill & Melinda Gates Foundation (BMGF) outside of the submitted work. ML and CM have received a supplemental grant from BMGF for preclinical and early clinical development of CFZ as a treatment for cryptosporidiosis (OPP1156296). KJ is a Wellcome International Training Fellow (Grant number 201945/Z/16/Z) and has received investigator-initiated grant support from GlaxoSmithKline Biologicals group of companies. DHn is a current employee of BMGF. WVV has patents issued for bumped kinase inhibitors (BKIs) for the therapeutic treatment of cryptosporidiosis diarrhoea and is a founder and has stock of ParaTheraTech LLC, a company that is developing BKIs for animal health indications. All other authors declare no competing interests.

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Novartis provided both the clofazimine and placebo. TechLabs provided *Cryptosporidium* rapid diagnostic and ELISA tests.

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Table 1. Baseline characteristics of participants

Characteristic	Part A CFZ group (n=12)	Part A placebo group (n=10)	Part B CFZ group (n=11)
Age, years	39.8 (±7.8)	39.1 (±12.0)	44.1 (±9.6)
Male sex (%)	8 (67%)	2 (20%)	7 (64%)
BMI, kg/m^2	16.3 (±1.7)	18.0 (±3.1)	18.9 (±1.4)
Pulse rate, beats/min	90.9 (±12.4)	95.9 (±14.9)	78.1 (±6.7)
Systolic blood pressure, mmHg	99.3 (±15.0)	106.4 (±16.5)	116.5 (±11.8)
Diastolic blood pressure, mmHg	68,3 (±10.1)	71.2 (±8.8)	75.7 (±12.3)
Hemoglobin, g/dL	10.6 (±2.2)	10.8 (±2.8)	14.0 (±1.3)

Hematocrit, %	32.3 (±6.5)	32.6 (±8.7)	42.3 (±3.6)
White blood cells, 10^9/L	2.9 (±1.4)	3.8 (±2.8)	5.0 (±1.7)
Neutrophils, 10^9/L	1.6 (±0.9)	2.1 (±2.1)	2.5 (±1.2)
Lymphocytes, 10^9/L	0.8 (±0.5)	1.1 (±0.7)	2.0 (±0.8)
CD4 absolute, cells/µL			
Mean (±SD)	25.3 (±24.4)	170.4 (±321.7)	422.0 (±231.3)
Median (IQR)	23.0 (8.0, 32.0)	22.5 (17.0, 86.0)	361.0 (216.0, 634.0)
HIV viral load, copies/µL	241,981.5 (±262,806.03)	679,025.13 (±929,116.49)	257.5 (±805.7)
ARV duration, days	1424 (±1547.6)	2011 (±1409.3)	1265 (±1810.3)
Blood urea nitrogen, mmol/L	4.9 (±2.5)	3.9 (±1.1)	3.8 (±1.0)
Creatinine, µmol/L	82.0 (±37.2)	56.0 (±15.9)	65.4 (±14.0)
Alanine aminotransferase, IU/L	34.0 (±20.3)	40.3 (±19.5)	38.9 (±21.4)
Aspartate aminotransferase, IU/L	50.6 (±16.4)	63.0 (±30.4)	50.7 (±18.3)
Electrocardiogram (ECG)			
Normal (%)	11 (92%)	10 (100%)	11 (100%)
Abnormal, not clinically significant (%)	1 (8%)	0 (0%)	0 (0%)

QTc interval, ms	421.7 (±14.2)	418.3 (±17.0)	409.7 (±21.6)
Cryptosporidium spp. (%)			
C. parvum	7 (58%)	4 (40%)	N/A
C. hominis	2 (17%)	1 (10%)	N/A
C. meleagridis	1 (8%)	3 (30%)	N/A
C. viatorum	1 (8%)	0 (0%)	N/A
Unknown[a]	1 (8%)	2 (20%)	N/A
Co-pathogens detected at diarrheagenic amount [14] (%)	8 (67%)	3 (30%)	N/A
Diarrhea duration,[b] days	17 (±7.6)	34 (±57)	N/A
Stool ELISA positivity (D1, %)	7 (58%)	2 (20%)	N/A
Log number of cryptosporidium shed in first collected stool of day, *Cryptosporidium* per gram stool (D-1)	13.9 (±2.7)	15.0 (±2.2)	N/A
Total daily cryptosporidium shedding, *Cryptosporidium* per gram stool (D-1)	22.3 (±2.9)	22.1 (±3.2)	N/A
Total stool weight, g (D-1)	320.3 (±214.6)	245.8 (±299.4)	N/A

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Most severe diarrhea severity ~~grade[b]~~ grade[c] (mild)	9 (75%)	3 (30%)	N/A
Stool consistency severity grade ≥3 (D-1, %)	9 (75%)	6 (67%)	N/A
Number of diarrheal episodes,[b, c] D1	1.3 (±1.1)	0.8 (±1.3)	N/A

ARV, antiretroviral therapy; BMI, body mass index; D, day; IQR, interquartile range; SD, standard deviation

All values are mean (±SD) unless otherwise listed.

[a]Failed to amplify on sequencing of 18s and gp60.

[b]Subjects with diarrhea duration entries '>2 weeks' were treated as 21 days for calculations of summary statistics.

[c]Observed over the first 24-hour dosing interval after the first study dose.

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Table 2. Comparison of pharmacokinetic parameters in Part A and B subjects

PK parameter		Part A (n=12)		Part B (n=11)	
		Mean (±SD)	% CV	Mean (±SD)	% CV
Day 1	C_{min} (ng/mL)	35.83 (±37.28)	323	74.74 (±24.51)	46
	C_{max} (ng/mL)	97.55 (±117.9)	195	193.3 (±93.50)	58
	T_{max} (h)	19.73 (±5.67)	-	14.776 (±7.537)	-
	AUC_{0-24} (ng.h/mL)	1364.0 (±1754.0)	219	2851.0 (±1256.0)	50
Day 5	C_{min} (ng/mL)	258.8 (±353.1)	187	455.8 (±221.5)	47
	C_{max} (ng/mL)	280.7 (±355.2)	173	514.1 (±202.0)	39
	T_{max} (h)	9.679 (±10.81)	-	6.683 (±3.765)	-
	AUC_{0-24} (ng.h/mL)	6863.0 (±8552.0)	172	11298.0 (±5580.0)	59

Summary	$t_{1/2}$ (h)[a]	336.5 (±84.71)	25	535.5 (±4.950)	1
	R_{AUC}	5.905 (±3.516)	57	4.111 (±1.579)	50

AUC, area under the curve; Cmax, peak plasma concentration; Cmin, trough plasma concentration; CV, coefficient of variation; R_{AUC}, accumulation ratio for AUC_{0-24} for Day 5 to Day 1; SD, standard deviation; Tmax, time to reach Cmax; $t_{1/2}$, elimination half-life

[a]Elimination half-life of clofazimine was previously found to be up to 70 days upon repeat dose administration~~,[26]~~ [27]; therefore the relatively short plasma sampling schedule in this study may not be accurately capture the $t_{1/2}$ parameter in these populations.

Table 3. Summary of adverse events

		Part A – CFZ (n=12)	Part A – placebo (n=10)	Part B (n=11)
Any solicited adverse event	Any severity	12 (100%)	10 (100%)	0 (0%)
	Max severity	2 (17%)	0 (0%)	0 (0%)
Abdominal pain	Any severity	8 (67%)	7 (70%)	0 (0%)
	Max severity	1 (8%)	0 (0%)	0 (0%)
Vomiting	Any severity	4 (33%)	4 (40%)	0 (0%)
	Max severity	1 (8%)	0 (0%)	0 (0%)
Diarrhea	Any severity	9 (75%)	4 (40%)	0 (0%)
	Max severity	0 (0%)	0 (0%)	0 (0%)

Anorexia	Any severity	4 (33%)	3 (30%)	0 (0%)
	Max severity	0 (0%)	0 (0%)	0 (0%)
Skin discoloration	Any severity	0 (0%)	0 (0%)	0 (0%)
Nausea	Any severity	5 (42%)	5 (50%)	0 (0%)
	Max severity	1 (8%)	0 (0%)	0 (0%)
Malaise	Any severity	6 (50%)	3 (30%)	0 (0%)
	Max severity	1 (8%)	0 (0%)	0(0%)
Urgency of defecation	Any severity	5 (42%)	4 (40%)	0 (0%)
	Max severity	0 (0%)	0 (0%)	0 (0%)
Any adverse events with fatal outcome		3 (25%)	1 (10%)	0 (0%)
Number of unsolicited adverse events		13	12	3
Subjects with at least one unsolicited adverse event		6 (50%)	4 (40%)	3 (27%)
Subjects with a serious adverse event		5 (42%)	2 (20%)	0 (0%)
Any unsolicited adverse event related to study drug		2 (17%)	0 (0%)	3 (27%)
Any unsolicited adverse event leading to discontinuation of study drug		0 (0%)	1 (10%)	0 (0%)

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