Chapter 19

In Vitro Culture of *Cryptosporidium parvum* Using Hollow Fiber Bioreactor: Applications for Simultaneous Pharmacokinetic and Pharmacodynamic Evaluation of Test Compounds

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Abstract

Hollow fiber technology is a powerful tool for the culture of difficult-to-grow cells. *Cryptosporidium parvum* has a multistage sexual and asexual life cycle that has proved difficult to culture by conventional in vitro culture methods. Here, we describe a method utilizing a hollow fiber bioreactor for the continuous in vitro growth of *C. parvum* that produces sexual and asexual stages. The method enables the evaluation of potential therapeutic compounds under conditions that mirror the dynamic conditions found in the gut facilitating preliminary pharmacokinetic and pharmacodynamic data to be obtained.

Key words Hollow fiber bioreactor, *Cryptosporidium parvum*, In vitro culture, Pharmacokinetic, Pharmacodynamics

1 Introduction

Despite the significant health risks and global distribution of cryptosporidiosis, there is no consistently effective therapy for the most-at-risk populations, that is, malnourished infants and immunocompromised individuals. Recent advances in manipulating the parasite genome [1] and chemotherapeutic profiling [2–4] are an encouraging sign that this bleak situation will change. Recent advances in continuous culture of *Cryptosporidium* has focused on developing a 3D culture system using adult murine colon cells [5] or novel bioengineered human intestinal cells [6] which has produced novel insights into parasite invasion and significantly extended the length of culture time compared to the 2D culture method. However, these techniques are limited in terms of parasite numbers obtained. The 3D models overcome the major obstacle associated with conventional 2D culture methods where host cells

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receive nutrients and oxygen from the apical surface (except for those systems that use porous membrane inserts like the Costar Transwell system); this is contrary to the in vivo situation where the enterocytes receive nutrients and oxygen from the basal surface and the apical surface faces the lumen of the gut. However, current intestinal implant models fail to provide the low oxygen environment present inside the gut lumen, which restricts the long-term growth of the parasite. The use of hollow fiber technology allows the creation of the biphasic environment present in the gut and overcomes many problems associated with the long-term culture of *C. parvum* [7, 8]. This protocol describes the establishment of hollow fiber bioreactors that can be used to simulate in vivo conditions by providing oxygen and nutrients to the basal surface of host intestinal cells that are attached to the outside of the hollow fibers (Fig. 1). The environment inside the reactor is adjusted to mimic the lumen of the gut hence the apical surface of the intestinal cells is established in a low redox, high-nutrient environment that favors high growth rates and long-term maintenance of *C. parvum*. The use of this method provides 10^8–10^9 oocysts which can be used for molecular and biochemical studies. It also has the advantage of avoiding the use of harsh chemicals such as potassium dichromate, which is used as a long-term storage medium at 4 °C and has the advantage of sanitizing the oocysts; and chlorine is currently used as both a sanitizer and to enhance excystation of oocysts obtained from animal sources [9].

2 Materials

All solutions are prepared using distilled, deionized water. The intracapillary space (ICS) medium, vitamin mix, and lipid mix is stored for a maximum of 6 months at −18 °C. The extracapillary space (ECS) medium and thiol mix is prepared fresh for each batch. The thiol mix is prepared using sterile distilled water that is boiled and cooled under a jet of nitrogen gas that is filtered using a 0.4 μm pore size filter.

2.1 Intracapillary Space Medium

To 1 L of Minimum Essential Medium (MEM) with l-glutamine and phenol red, without HEPES add 0.058 g heparin, 0.29 g l-glutamine, 23.8 g 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) pH 7.8 with 5 M NaOH, 4.5 g d-glucose, 0.035 g ascorbic acid, 0.04 g p-aminobenzoic acid, 0.02 g Ca pantothenate, 0.001 g folic acid, and 100 mL horse serum. The base ICS medium is filter-sterilized using sterile disposable 0.2 μm bottle top filters with a polyethersulfone (PES) membrane.

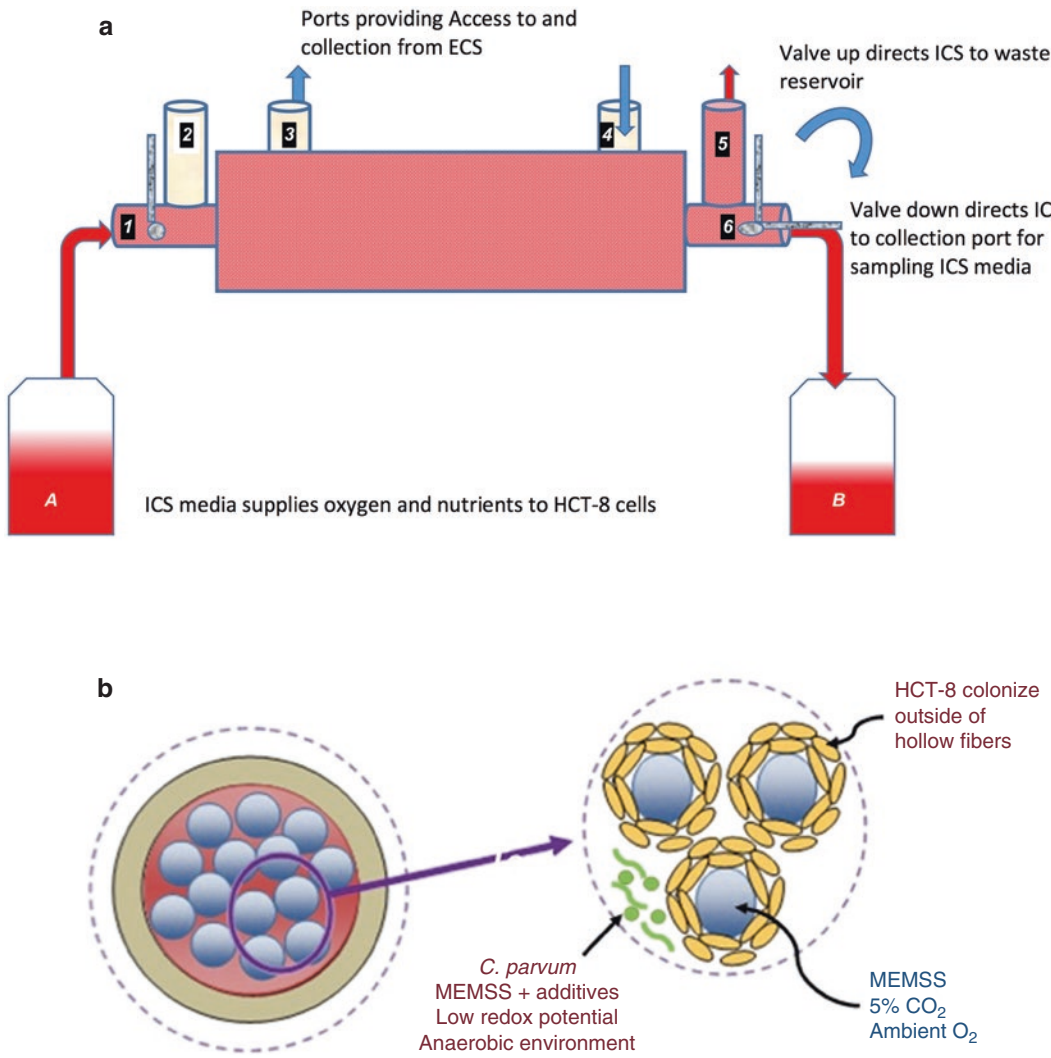

Fig. 1 Hollow fiber bioreactor (HFB) modified for pK/pD analysis of test compounds. (**a**) MEM containing 10% horse serum is placed in the container on the left and pumped through port [1] into the hollow fibers providing oxygen and nutrients for the HCT-8 cells. Test compound is added to the ECS via port [3] and mixed by alternating depressing of syringes attached to ports [3, 4]. Test compound that is transported through the HCT-8 cells and excreted into the ICS is collected by diverting the waste from port [6] to port [5]. (**b**) Section through the HFB showing ICS (blue circles) and ECS (red). Host HCT-8 cells (yellow) attach and grow around the hollow fibers, while parasite stages (green) develop in the ECS

2.2 Extracapillary Space Medium

To 10 mL of Minimum Essential Medium (MEM) with l-glutamine and phenol red, without HEPES, add 0.15 g Na taurodeoxycholate, 0.005 g Na thioglycolate, and 0.055 g mannitol. The thiol mixture is prepared by mixing 0.2 g of each of reduced glutathione, taurine, betaine, and cysteine in a glass vial containing 10 mL of distilled water, that has been boiled and cooled under a 0.4 μm filtered stream of nitrogen gas. The thiol mix is stored under a nitrogen

atmosphere in 2-mL borosilicate glass vials (screw caps with septa). The thiol mix (0.15 mL) is aseptically added to 9 mL of the ECS medium in a sterile hood.

A lipid mix is prepared by adding 0.067 g of oleic acid and 0.18 g of cholesterol (water-soluble) to 10 mL of sterile distilled water. The lipid mix (0.2 mL) is added to 9.15 mL of the base mix to produce the ECS working medium, and brought to a pH of 7.8. The medium is sterilized using a 0.22-μm syringe driven filter with PES membrane.

2.3 Host Cells—HCT-8 Cell Line

Human adenocarcinoma intestinal epithelial cells are grown to confluence in MEM with l-glutamine and phenol red, without HEPES using 75 cm^2 "tissue culture" treated cell culture flasks in a 5% CO_2 incubator at 37 °C. HCT-8 cells are collected by replacing the growth medium with 2 mL of trypsin– EDTA (0.25%), phenol red solution, and incubating at 37 °C for 15 min. The trypsin is removed by centrifugation at 4000 × *g* for 5 min, and the HCT-8 pellet gently resuspended in MEM to a density of 10^6 cells per mL of ICS mix.

2.4 C. parvum

C. parvum oocysts (Bunch Grass Farm, Deary, ID) sterilized by immersion in 10% diluted commercial bleach (~5% hypochlorite) at 4 °C for 5 min are washed with a tenfold volume of distilled water six times and enumerated using a hemocytometer. Oocysts are resuspended to 10^5 per mL in the ECS medium.

2.5 Test Compound

Bump kinase inhibitor BKI-1294 (Fig. 2) was provided by the Center for Emerging and Reemerging Infectious Diseases (CERID), Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington (Seattle, WA, USA). The compound is stored as a 20 mM stock solution in dimethyl sulfoxide at −20 °C and diluted to a working solution of 0.512 mM by adding 102 μL of the stock solution to 2 mL of dimethylsulfoxide (DMSO) and brought to a final volume of 4 mL in sterile distilled water. Details of the extraction and liquid chromatography mass spectrometry (LC MS/MS) analysis of BKI-1294 in samples collected from the bioreactor are provided by Hulverson et al. [10].

2.6 Analysis of C. parvum Growth

C. parvum growth is determined by analysis of the amount of *C. parvum* 18S-rRNA present compared to the HCT-8 18S-rRNA as previously described [11]. Briefly, this was performed using 0.5 mL samples collected from the ECS after centrifugation at 14,000 × *g* for 5 min. Gently resuspend the pellet in 100 μL of iScript buffer and subject to six freeze/thaw cycles comprising 1 min in liquid nitrogen followed by 1.5 min at 70 °C. Centrifuge the sample at 16,162 × *g* for 5 min and remove the lysate. Add 1 volume of 70% ethanol, and transfer the sample to an RNeasy Mini spin column

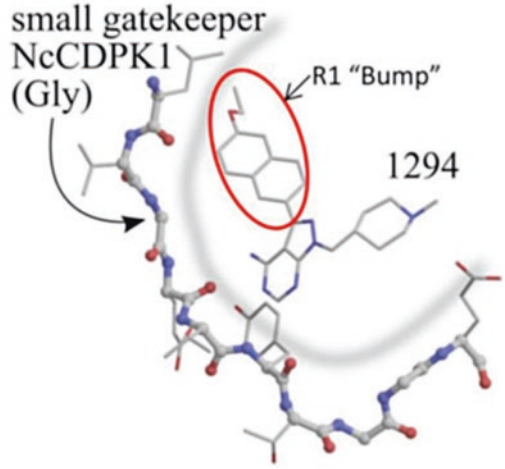

Fig. 2 Structure and mechanism of Bumped Kinase inhibitor 1294. BKI-1294 was designed specifically as a *Toxoplasma gondii* cyclin dependent protein kinase 1 (CDPK1) inhibitor. A key structural difference between parasite and human CDPKs occurs at the gatekeeper residue in the adenosine triphosphate (ATP)-binding pocket which contains a small glycine residue at this position, compared to human kinases that have larger amino acid residues in this position. A pyrazolopyrimidine scaffold, comprising a 6-alkoxy-2-naphthyl group at the C-3 position binds in the ATP-binding pocket. This bulky C-3 substituent is sterically hindered by the larger gatekeeper residues found in human kinases. A 4-piperidinylmethylene group at the N-1 position fully occupies the ribose-binding pocket in many parasites and forces the bulky C-3 group into a position that cannot be accommodated by human kinases

(Qiagen, Inc., Germantown, MD) placed in a 2 mL collection tube and centrifuge for 15 s at 8000 × *g*. Discard the flow-through and follow the manufacturer's instructions (iScriptTM, RT-qPCR sample preparation kit, Bio-Rad Laboratories, Hercules, CA). *C. parvum* is enumerated by quantitating the *Cp*18S-rRNA using the following specific forward and reverse primers - *Cp*18S-995F: 5′-TAGAGATTGGAGGT TCCT-3′ and *Cp*18S-1206R: 5′-CTCCACCAACTAAGAACGCC-3′, and comparing it to the human *Hs*18S-rRNA using the following primer sets - *Hs*18S-F1373: 5′-CCGATAAC GAACGAGACACTCTGG-3′ and *Hs*18S-R1561: 5′-TAGGGTAGGCACACGCTGAGC C-3′. The amount of 18S-rRNA is determined in 5 ng samples by RT-qPCR as described by the manufacturer using a Luna Universal Probe One-Step® RT-qPCR kit (New England BioLabs, Ipswich, MA) and a Quant Studio 6 Flex 44 instrument (Applied Biosystems Inc., Beverly, MA). The cell number is calculated from the C_T means using a set of standards prepared from 10^4, 10^5, and 10^6 *C. parvum* oocysts and compared to 10^4, 10^5, and 10^6 HCT-8 cells.

2.7 Microscopic Evaluation of C. parvum Stages

Dual stain *C. parvum* samples (50 μL) are prepared by the addition of 20 μL each of a fluorescein-labeled mouse monoclonal antibody to the oocyst outer wall protein (Crypt-a-Glo™, Waterborne Inc., New Orleans, LA) and a fluorescent polyclonal antibody to

sporozoites and other motile stages (Sporo-Glo™, Waterborne Inc., New Orleans, LA). Samples are incubated at room temperature in the dark for 30 min and excess antibody is removed by centrifugation at 14,000 × *g* for 3 min. Samples are gently resuspended with 40 μL of phosphate buffered saline (PBS) and examined microscopically using a fluorescent microscope at 400× magnification. Crypt-a-Glo™ stained oocysts are visualized using an excitation wavelength of 410–485 nm and an emission wavelength of 515 nm; Sporo-Glo™ stained motile stages are visualized using an excitation wavelength of 535–550 nm and an emission wavelength of 580 nm.

3 Methods

3.1 Preparation of the Bioreactor

1. The culture reservoir bottles are prepared by loosely attaching a 33 mm reservoir cap and tubing (included with the cartridge) into a 125 mL bottle. Loosely wrap the Luer fittings on the end of the reservoir cap tubing with foil and place the entire assembly into an autoclave bag and seal.
2. Autoclave at 121 °C, 15 lb./in^2 for 20 min (high vacuum setting).
3. Inside a biosafety cabinet, remove the 20 kD MWCO polysulfone fiber cartridge (FiberCell Systems, New Market, MD) from its packaging and check the Luer fittings between the hollow fiber module and the flow path to ensure that they are finger tight.
4. The 20 kD MWCO polysulfone fiber cartridge has additional ports for access to the intracapillary space (ICS) as well as the extracapillary space (ECS) and an ECS volume of 3.2 mL, making it suitable for testing experimental compounds. For production of large parasite numbers, the 20 kD MWCO medium cartridge (FiberCell Systems) with an ECS volume of about 20 mL is recommended. However, the medium cartridge lacks additional ports to access the ICS and the larger ECS volume requires the use of five times more compound, making it unsuitable for pK/pD studies.
5. Remove the foil from one of the Luer fittings on the 33 mL reservoir cap and attach to the inlet flow path on the cartridge. Spray with 70% ethanol and wipe with an alcohol pad. Apply ½ turn counter rotation to the tubing prior to attachment to prevent kinking of the tubing. Attach the other Luer fitting to the outlet flow path. Check fittings to ensure that they are tightly attached.

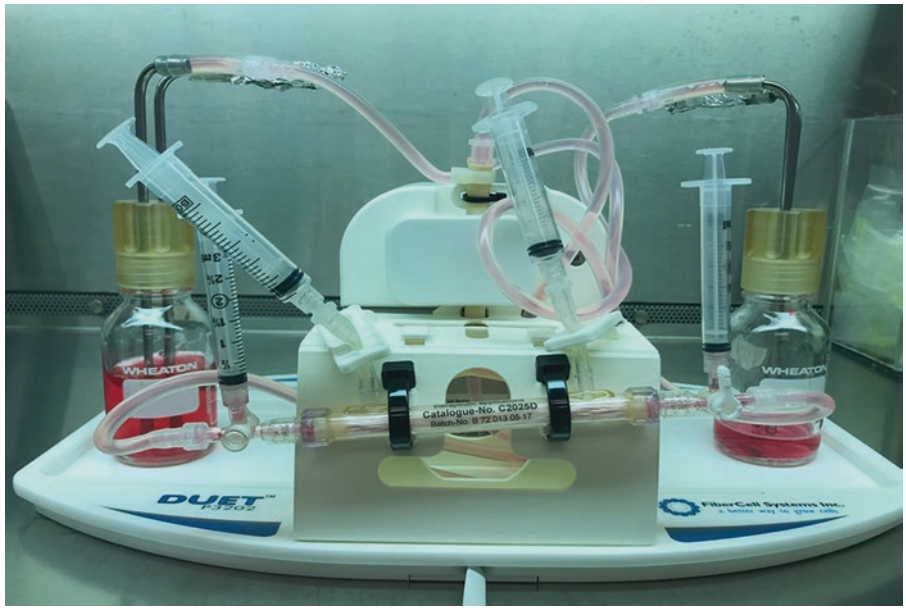

Fig. 3 Hollow Fiber Bioreactor pK/pD set up

6. Connect the cartridge to the Duet Pump (FiberCell Systems) and ensure that the lines are properly engaged in the pump housing (Fig. 3).

3.2 Preculture

1. When working with adherent cells, the cartridge should be washed with two volumes of 125 mL of sterile PBS each for 24 h prior to filling the cartridge with 125 mL of cell culture medium.
2. Ensure that the left and right end port side clamps are open.
3. Aseptically fill 100 mL of sterile PBS into 125 mL bottle attached to cartridge using the 33 mm caps (the flow path and cartridge for medium cartridge holds about 30 mL of media). Prime the pump manually using the thumb and forefinger until all the air is pushed out of the tubing then close the left and right end port side clamps.
4. Attach a sterile syringe containing 30 mL sterile PBS to the left extracapillary space (ECS) port and attach an empty sterile syringe to the right ECS port.
5. Open the clamps on the left and right ECS side ports and push the sterile PBS into the ECS. Tilt cartridge upwards and fill medium taking care to remove all air present in the ECS. If the volume is not sufficient, repeat with more sterile PBS.
6. Close the left and right ECS side ports, spray with 70% alcohol and remove the syringes, spray with 70% alcohol and wipe with

an alcohol pad prior to attaching clean sterile 3 mL syringes to the ports. Open the left- and right-side port clamps.

7. Connect the hollow fiber cartridge to the Duet pump unit in a 37 °C, 5% CO_2 incubator. Set a medium flow rate of 5, leave for 24 h.
8. After 24 h, the left and right end port clamps are closed and the PBS reservoir bottle is aseptically changed for a fresh 125 mL bottle containing 100 mL of sterile PBS and returned to the incubator for 24 h.
9. Aseptically fill a sterile 125 mL reservoir bottle with 100 mL of MEM media.
10. Leave the reservoir cap loose by ½ turn. Attach a sterile syringe containing 30 mL of MEM to the left ECS port and an empty 60 mL syringe to the right ECS port.
11. Open the clamps on the left and right ECS side ports and push the sterile MEM into the ECS. Tilt cartridge upwards and fill medium taking care to remove all air present in the ECS. If the volume is not sufficient, repeat with fresh MEM.
12. Close the left and right ECS side ports, spray with 70% alcohol and remove the syringes, spray with 70% alcohol and wipe with an alcohol pad prior to attaching clean sterile 3 mL syringes to the ports.
13. Open the left and right ICS side port clamps (Fig. 1). Connect the hollow fiber module to the Duet pump unit in a 37 °C, 5% CO_2 incubator. Set a medium flow rate of 5 and leave overnight.
14. Repeat using MEM plus 10% horse serum.

3.3 Addition of Host Cells

1. Close the left and right ICS end port slide clamps of the cartridge.
2. Into a 10 mL sterile syringe take up 5 mL of HCT-8 cells (5×10^6) suspended in ICS mix.
3. Spray the left and right ECS side port Luer connections with 70% ethanol and wipe with an alcohol pad.
4. Replace both 3 mL syringes on the ECS ports with the syringe containing the HCT-8 cells on the left ECS port and an empty 10 mL syringe to the right ECS side port.
5. Open the ECS side port clamps.
6. *Gently* flush the cell suspension through the ECS back and forth between the two syringes three to five times, to ensure equal distribution of the cells.
7. When finished, both syringes should contain an equal volume (2.5 mL in each syringe).
8. Loosen the reservoir cap ½ turn, close the left ECS end port slide clamp, open the right-hand ICS end port slide clamp (this

allows excess medium to flow into the reservoir bottle), and empty the syringe on the right hand side into the ECS (be careful not push air into the column).

9. Close the right ECS side port slide clamp, open the left ECS clamp, and empty the syringe on left hand side into the ECS.
10. Close all the side port slide clamps and spray with 70% ethanol and use a sterile alcohol swab before replacing both syringes with new 3 mL sterile syringes.
11. Tighten the reservoir cap. Open the left and right ICS end port slide clamp. Place the cartridge into the Duet pump inside a 37 °C, 5% CO_2 incubator, and set flow rate to 5. After 3 days, increase the flow rate to 10.
12. Change the MEM plus 10% horse serum medium every 24 h and the check the glucose concentration using a digital glucose meter (typically used for blood glucose monitoring).
13. The glucose concentration of the MEM plus horse serum medium should be 75 mg/dL. When the glucose concentration falls to approx. 50% (30 mg/dL) of its starting concentration, the bioreactor is inoculated with *C. parvum*, at which time the MEM plus horse serum is changed for the complete ICS medium mix.

3.4 Inoculation of (HFB) with Parasites

1. Treat 10^5 *C. parvum* oocysts with 10% (v/v in distilled water) diluted commercial bleach and wash seven times with 30 mL of sterile distilled water, prior to suspending in 3 mL of MEM containing the ECS supplements described in the Materials section.
2. Close the left and right ICS end port clamps.
3. Spray the left and right ECS ports with 70% alcohol, and remove the left ECS syringe using an alcohol swab.
4. Attach the syringe containing the 3 mL of *C. parvum* oocysts to the left ECS port, and a clean sterile 3 mL syringe to the right ECS port.
5. Open the slide clamps to the left and right ECS ports and slowly push the oocysts into the cartridge, while gently pulling up on the right syringe.
6. Gently mix the contents of the ECS by alternating filling of the right and left syringes approximately five times, *caution should be taken not to introduce air into the cartridge*. With both left and right ECS syringes containing equal volumes (approx. 1.5 mL), close the right ECS port clamp, open the right ICS end port slide clamp, and push down on the syringe attached to left ECS port until the contents have been added to the ECS, *avoid adding air into the cartridge*.

7. Slide the left ECS port clamp to close and open the right ECS slide clamp and push down on the syringe until the contents of the syringe are added, *avoid adding air into the cartridge*.
8. Slide the right ECS port clamp closed (*see* **Note 1**).
9. Open the left ICS end port and spray the left and right ECS ports with 70% alcohol and using an alcohol pad replace the syringes on the left and right ECS ports with clean sterile 10 mL syringes.
10. Replace the reservoir bottle with 125 mL of the complete ICS medium mix, return the unit to the incubator, and set the flow rate to 10.
11. The pH and glucose in the ICS medium are monitored every 48 h, by removing 5 mL directly from the reservoir bottle. When the glucose concentration falls to undetectable levels in 48 h, the reservoir is changed for a 500 mL bottle of the complete ICS medium mix.
12. Samples (0.5 mL) are collected and examined microscopically for parasites and 0.25 mL used for RT-qPCR analysis of parasite numbers as described in Materials, Subheading 2.7.

3.5 pK/pD Arrangement

1. After 7–10 days in culture the ICS inlet is connected to a fresh bottle containing 600 mL of media and 0.5 mL of the ICS media removed for use as a starting time point.
2. Connect the ICS outlet to an empty sterile flask to prevent recirculation of the media.
3. Remove 0.5 mL from the ECS for microscopic examination and enumeration of parasites by spraying with 70% ethanol and attaching a clean 3 mL syringe containing 0.5 mL of ECS media to the left ECS port, and an empty clean sterile 3 mL syringe to the right ECS port.
4. Depress the left syringe to displace 0.5 mL of the ECS to the right hand syringe.
5. Gently alternate pressure on the syringes about five times to mix the contents, and end with the left syringe containing ECS sample and the right syringe empty.
6. Close the left and right ECS ports and remove the left ECS syringe containing the ECS sample and keep for analysis.

3.6 pK/pD: Addition of Test Compound

1. Spray the left sampling port with 70% alcohol and attach a fresh sterile 3 mL syringe containing 0.5 mL of 0.512 mM of BKI-1294.
2. Add the test compound as previously described for addition of parasites (Subheading 3.4) but end with 0.25 mL of the ECS medium in both left and right syringes.

3. Close the right ECS port and make sure the left ICS port is closed, with the right ICS port open, depress the syringe on the left ECS port, *do not to introduce an air bubbles.*
4. Close the left ECS port and open the right ECS port and depress the syringe on the right, again ensure that *no air bubbles are introduced to the cartridge.*
5. Close the right ECS port and spray with 70% ethanol before replacing both the left and right ECS ports with new sterile 3 mL syringes.
6. The right and left ICS ports are turned to open and the unit placed back into the incubator at a pump setting of 0.5 mL/min.

3.7 Collecting Samples for Pharmacokinetic/Pharmacodynamics Analysis

1. At 1 h intervals for the first 4 h, collect 0.5 mL samples from the ICS using the sampling port on the right-hand side that samples the ICS medium directly exiting the hollow fibers that contains any test compound taken up and excreted by the host cell epithelial layer (Fig. 1).
2. ICS samples are collected by attaching a sterile 3-mL syringe to the sampling port via the Luer lock fitting (Fig. 1) and the lever turned from the upright position to the right which directs the ICS media to the syringe. The left ICS sampling port should remain open (lever points up) to prevent pulling fluid into the ICS from the ECS.
3. The ICS sampling port is returned to open (lever up) after sampling, and the cartridge returned to the incubator.

3.8 pK/pD: Days 2 to 4

After 24 h, sample 0.5 mL from the ECS to quantitate the parasite load as described in Subheading 3.5. Add 0.5 mL of 0.512 mM of the bump kinase inhibitor BKI-1294 as described in Subheading 3.6; and sample 0.5 mL from the ICS at 1 h intervals for 4 hours as described in Subheading 3.7.

3.9 Analysis of Bump Kinase Inhibitor 1294

Test compounds can be administered by addition to the ICS, equivalent to serum dosing, or by addition to the ECS, equivalent to oral dosing. Cryptosporidiosis is an intestinal disease and hence delivery via the ECS port was selected. The half-maximal effective concentration (EC_{50}) for the bumped kinase inhibitor BKI-1294 was previously determined to be 2.65 μM [9]. Preliminary experiments were designed to determine a dose that would achieve a sustained EC_{50} concentration for the treatment period. The method described permits evaluation of both the concentration of BKI-1294 required to completely inhibit cryptosporidial growth in the culture and the time required to achieve this goal. Based on an EC_{50} of 2.7 μM and gastrointestinal BKI concentrations observed with an efficacious oral dose in mice, a 3- or 4-day dosing regimen of 20 μM, 40 μM, or 80 μM BKI-1294 was evaluated [2].

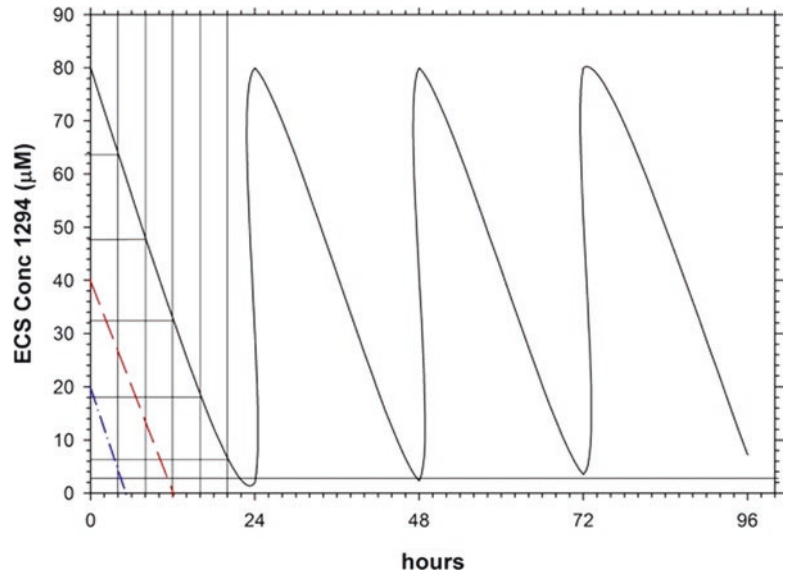

Fig. 4 Pharmacodynamics of 1294 in the ECS during a 4-day dosing schedule of 80 μM per day. BKI-1294 decreases exponentially in the ECS over 24 h, but remains above the EC_{50} of 2.65 μM for 22 h. Subsequent doses on days 2–4 show that BKI-1294 remains above the ECS for 24 h. Predicted doses of 40 μM (----) and 20 μM (---) will fall below the EC_{50} in 12 and 6 h, respectively

The BKI-1294 concentration in the ECS following a 20 μM and 40 μM daily dose revealed that exposure to the EC_{50} concentration was ≤4 h and 11 h, respectively (Fig. 4), and parasite growth although reduced by 2 and 3 logs was not completely inhibited and recovered regaining the starting numbers after 4 days posttreatment for the 20 μM dose (Fig. 5), and 8 days posttreatment for the 40 μM dose (not shown).

The BKI-1294 concentration in the ECS following an 80 μM daily dose revealed an ECS exposure equal to or greater than the EC_{50} for the first 22 h; subsequent doses of 80 μM every 24 h provides an EC_{50} exposure for ≥24 h (Fig. 4).

The pharmacokinetics of BKI-1294 following an 80 μM per day for 4 days experiment reveal that the concentration of BKI-1294 in the ICS, equivalent to absorption and excretion of the

Fig. 5 (continued) of 8×10^6 parasites by day 4. (**b**) Three-day dosing with 80 μM BKI-1294. BKI-1294 was dosed on days 1–3, and parasite numbers were evaluated by qRT-PCR daily out to 37 days. Parasites had an initial 4-log reduction in growth compared to controls, and continued to slightly decrease up to 5 days past the last dose. However, parasite numbers gradually recovered and by day 23, regained control numbers. (**c**) Four-day dosing with 80 μM BKI-1294. BKI-1294 was dosed on days 1–3, and parasite numbers were evaluated by quantitative polymerase chain reaction (qRT-PCR) daily out to 30 days. Parasites had a 6-log reduction in growth compared to controls by day 5, and continued to decrease reaching undetectable numbers by 5 days past the last dose. Parasite numbers did not recover out to 26 days post the final dose

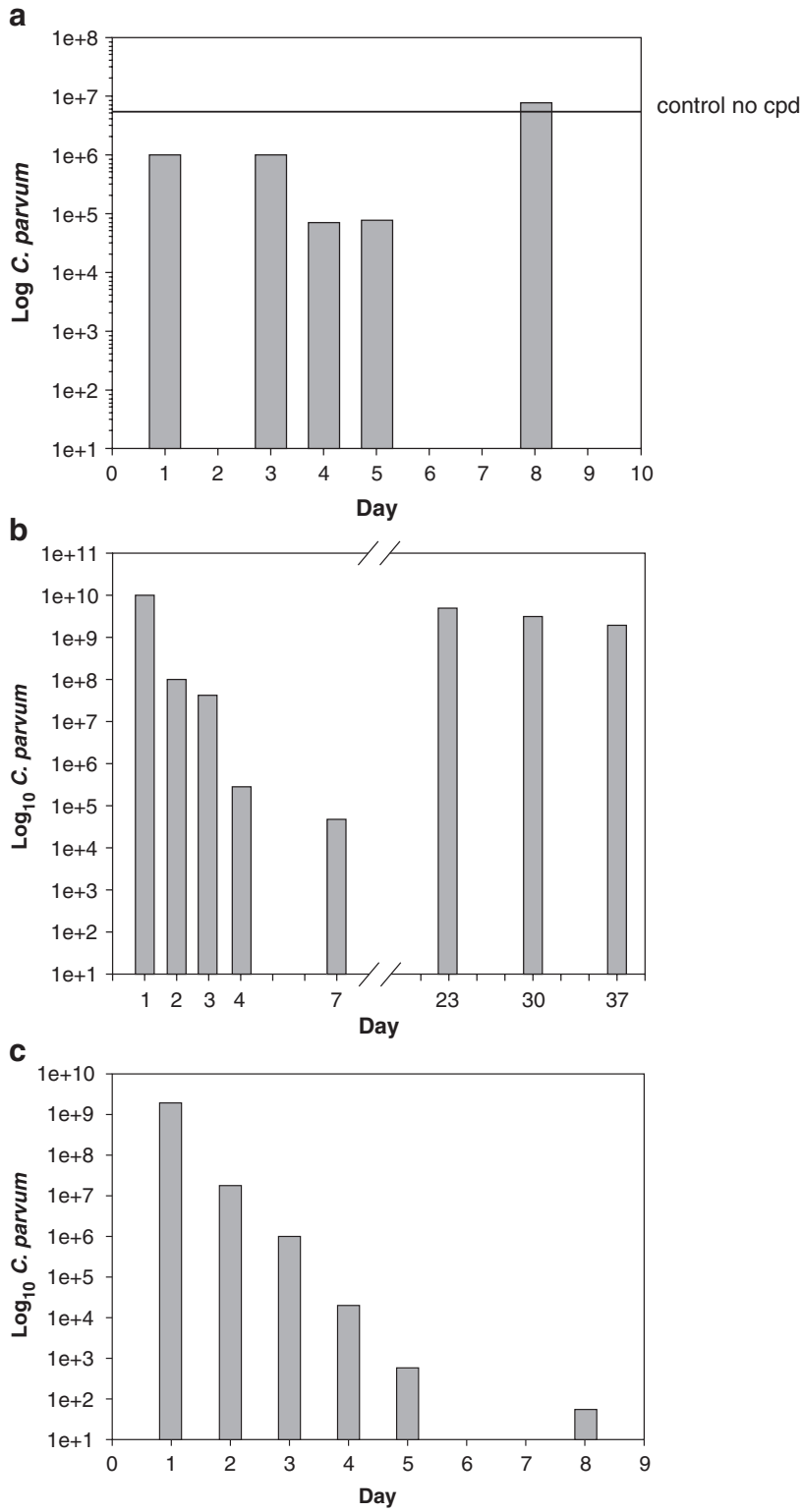

Fig. 5 Growth of C. parvum during treatment with BKI-1294. (**a**) Four-day dosing with 20 μM BKI-1294. BKI-1294 was dosed on Days 1–4, and parasite numbers were evaluated by qRT-PCR daily out to 8 days, and no sample was removed on day 2. Parasites had an initial 1-log reduction in growth compared to controls, but recovered to control numbers 4 days past the final dose. A control bioreactor receiving only the vehicle produced an average

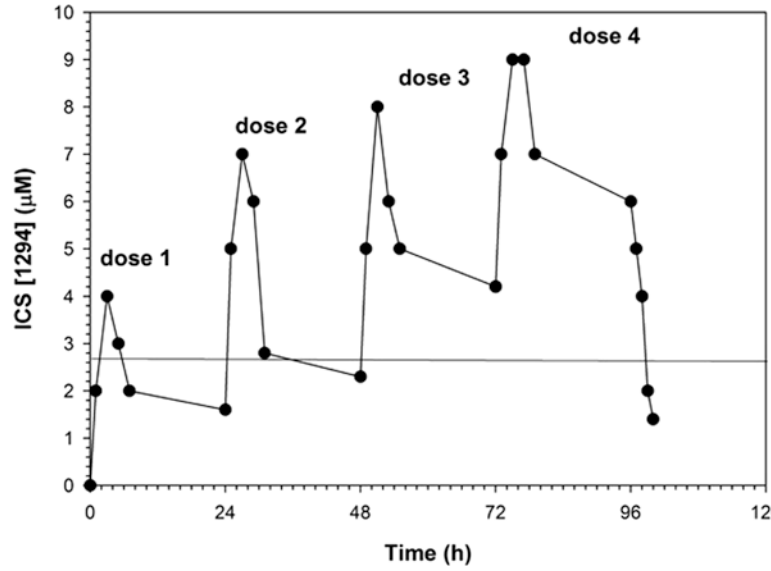

Fig. 6 Concentration of BKI-1294 in the ICS. The concentration of BKI-1294 in the ICS of an 80 μM daily dosed HFB once daily for 4 days (corresponding to changes in BKI-1294 concentration in the ECS shown in Fig. 4). ICS samples were collected from port 5 (Fig. 1) on an hourly basis for 4 h post dosing. The concentration of BKI-1294 in the ICS increases during the first 2 h post dosing and then gradually falls to below the EC50 after 6 h on day 1, and 12 h on day 2. From day 3 on the concentration of BKI-1294 in the ECS remains above the parasite EC_{50}

compound across the intestinal epithelium into the plasma, showed a transient increase for the first 2 h after addition to the ECS and then demonstrated an exponential decrease to approximately 1.5 μM BKI-1294 (Fig. 6). Subsequent doses of BKI-1294 had similar decay profiles in the ICS, but maintained a concentration at or above 2.5 μM. The 4-day treatment with 80 μM BKI-1294 did not show parasite recrudescence post 20 days of the final dose (Fig. 5). However, a 3-day exposure to 80 μM is insufficient for complete removal of parasites (Fig. 5). The results obtained using the modified HFB method agree with the in vivo data where a higher than expected dose, 60 mg/kg per day for 5 days, to cure a neonatal mouse model *C. parvum* infection resulted in a 2-log reduction of parasites but failed to cure [12].

The method is a valuable addition to the tools available for the determination of test compounds for the treatment of cryptosporidiosis. It has advantages over the standard 48 h in vitro assay in static multiwell plates where drug concentration does not vary over time as it does in vivo. It can be included in any compound screening method to advance compounds selected from the preliminary EC_{50} screen to provide valuable information concerning bioavailability and dosing schedules prior to in vivo analysis in animal

models thereby reducing the number of laboratory animals required for preliminary screening during drug development strategies.

4 Notes

1. When inoculating the cartridge initially, the user should experience minimal backpressure, after several months (>6 months) of continuous culture, an increase in backpressure can be experienced with the 3 mL ECS cartridge which can become significant hence it is not recommended for studies beyond this point.

Acknowledgments

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