Emerging treatment options for cryptosporidiosis

Melissa S. Love\textsuperscript{a} and Robert K.M. Choy\textsuperscript{b}

**Purpose of review**
Substantial progress has been made recently on the development of new therapeutics for cryptosporidiosis, an infection by the protozoan parasite Cryptosporidium that is associated with diarrhea, malnutrition, growth stunting, cognitive deficits, and oral vaccine failure in children living in low-resource settings.

**Recent findings**
Various drug discovery approaches have generated promising lead candidates. The repurposed antimycobacterial drug clofazimine was tested in Malawian HIV patients with cryptosporidiosis but was ineffective. Target-based screens identified inhibitors of lysyl-tRNA synthetase, phenylalanyl-tRNA synthetase, methionyl-tRNA synthetase, and calcium-dependent protein kinase 1. Phenotypic screens led to discovery of a phosphatidylinositol 4-kinase inhibitor, the piperazine MMV665917, and the benzoxaborole AN7973. The relationship between pharmacokinetic properties and in-vivo efficacy is gradually emerging. A pathway to clinical trials, regulatory approval, and introduction has been proposed but additional work is needed to strengthen the route.

**Summary**
Several lead compounds with potent activity in animal models and a favorable safety profile have been identified. A sustained effort will be required to advance at least one to clinical proof-of-concept studies. The demonstrated risk of resistance indicates multiple candidates should be advanced as potential components of a combination therapy.

**Keywords**
cryptosporidiosis, Cryptosporidium, diarrhea, drug development, low-resource settings

**INTRODUCTION**
Infection by the protozoan intestinal parasite Cryptosporidium spp. is a leading cause of diarrheal disease morbidity and mortality [1,2]. Recent estimates suggest Cryptosporidium spp. is responsible for approximately 7.6 million cases and between 48 000 and 202 000 deaths annually among young children in low-resource settings [3,4]. Beyond acute morbidity and mortality, both symptomatic and asymptomatic infections are associated with long-term sequelae including growth stunting, malnutrition, and cognitive development deficits [5,6]. Sporadic outbreaks from contaminated water supplies are common even in high-income countries [7]. Cryptosporidium hominis and anthropoontic Cryptosporidium parvum are most commonly identified in humans; person-to-person is the predominant mode of transmission [8], although zoonotic species are occasionally found in humans.

Despite the substantial disease burden caused by Cryptosporidium, treatment options remain limited. The only treatment approved by a stringent regulatory authority (the US Food and Drug Administration) is nitazoxanide, a drug that is efficacious in otherwise healthy adults but has marginal efficacy in malnourished children and is no better than placebo in immunocompromised HIV-positive patients [9]. Another limitation is that nitazoxanide is not approved for children younger than 12 months, the most vulnerable patient population. In the absence of highly effective specific therapy and a simple, point-of-care diagnostic test [10], in most low-resource settings, cryptosporidiosis is treated symptomatically with either oral or intravenous rehydration. A target product profile and use-cases of an ideal anticryptosporidial therapeutic have been proposed [11,12**].

\textsuperscript{a}Calibr, a Division of The Scripps Research Institute, La Jolla, California
\textsuperscript{b}PATH, Seattle, Washington, USA

Correspondence to Robert K.M. Choy, PATH, 2201 Westlake Avenue Suite 200, Seattle, WA 98121, USA. Tel: +1 206 285 3500; e-mail: rchoy@path.org

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As *Cryptosporidium* emerged as a major contributor to the global diarrheal disease burden in the past decade, a number of academic and industrial groups have advanced promising new chemical entities (NCEs) through a rigorous screening cascade (Fig. 1). The past 2 years have seen substantial progress with development of many NCEs for cryptosporidiosis, thus the purpose of this review is to provide an overview of these projects.

**PIPELINE OF EMERGING CRYPTOSPORIDIOSIS THERAPEUTICS**

In this review, we discuss recent advancements around seven candidates in development as cryptosporidiosis-specific therapeutics. These compounds emerged from either a target-based approach of a known or validated target in other pathogens, or a phenotypic whole-cell screen against *Cryptosporidium* parasites with a curated compound library (Table 1).

**Candidates from target-based screens**

Although target-based therapeutic screening has historically been perceived to be challenging in *Cryptosporidium* as it appropriates many essential metabolites from host cells, a number of promising candidates have emerged from target-based screens. One class is inhibitors of aminoacyl-tRNA synthetases (aaRSs) – enzymes that charge amino acids for polypeptide synthesis, and thus tend to be essential for growth. Three will be considered in detail below, along with calcium-dependent protein kinase 1 (CDPK1).

**Lysyl-tRNA synthetase**

Lysyl-tRNA synthetase (KRS) was originally identified as the target of cladosporin in *P. falciparum*. On the basis of structural homology, *P. falciparum* KRS (PfKRS) inhibitors were tested on *C. parvum* KRS (CpKRS) and a consortium of investigators led by University of Dundee recently published a comprehensive study of early lead compounds (subsequently referred to in this review as ‘leads’) targeting both enzymes [13]. A potent chromone compound was reported with excellent drug-like properties (Table 1). Because of toxicity observed in rodents, this early lead was deprioritized; however, it provided important validation of KRS as a drug target. Subsequent chemical refinement has identified further optimized leads with greater potency and less toxicity that will be tested in the neonatal calf efficacy model (B. Baragaña, personal communication). The early lead chromone has high solubility and oral bioavailability (Table 1), which likely contribute to its high systemic exposure. However, a later lead compound retained efficacy in mouse models with relatively lower systemic exposure. The basis for this disconnect is under investigation. Molecular modeling comparing PfKRS and CpKRS with human KRS suggested a potential mechanism for selectivity, which would reduce risks of on-target toxicity.

**Phenylalanyl-tRNA synthetase**

Like KRS, Phenylalanyl-tRNA synthetase (PheRS) was also initially validated as a drug target for *P. falciparum* and then subsequently explored as a target for *Cryptosporidium* by a consortium of academic and industrial collaborators. In an effort led by the Broad Institute, a curated set of compounds with previously established antimicrobial activity and known mechanisms of action (MoA) was screened, and a bicyclic azetidine with potent activity against *C. parvum in vitro* (Table 1) was identified [14*]. Homology with PfPheRS was beneficial in rapidly establishing a structure–activity relationship for CpPheRS inhibitors and identifying a range of compounds with varying pharmacokinetic and physiochemical properties, including differences in oral bioavailability, volume of distribution, metabolic half-life, and solubility. Several compounds with a range of properties were then tested in vivo using the NOD SCID gamma (NSG) mouse model and a direct relationship between higher bioavailability and greater in-vivo activity was demonstrated. As a confirmation of the MoA of bicyclic azetidines on PheRS, CRISPR-Cas9 was used to introduce a resistance-conferring mutation found in PfPheRS into CpPheRS, which afforded resistance to BRD7929, a representative compound.

**Methionyl-tRNA synthetase**

Exploration of Methionyl-tRNA synthetase (MetRS) inhibitors for cryptosporidiosis arose from a
program at the University of Washington investigating this enzyme as a target for other pathogens including *Trypanosoma brucei*. Crystal structures of the *Tb*MetRS bound to inhibitors were leveraged to identify the homologous binding sites in *C. parvum* and *C. hominis* MetRS, which retain 76% identity to *T. brucei*. Several *Tb*MetRS inhibitors were shown to potently inhibit *Cp*MetRS *in vitro* and suppress oocyst shedding in both the IFNγ KO and NSG mouse models [15*]. Pharmacokinetic analysis of *Cp*MetRS inhibitors found the compounds with highest activity in mouse models had both high systemic exposure (Table 1) and high levels in feces (>10 μmol/l), therefore, it was not possible to discern whether systemic or intestinal luminal exposure is more important for activity with this compound class. In the neonatal calf efficacy model [16], lead compound 2093 initially strongly
| Lead | Development phase | Compound | In-vitro activity | In-vivo efficacy | Pharmacokinetic profile |
|------|-------------------|----------|-------------------|-----------------|------------------------|
|      |                   | C. parvum | C. hominis | Enzymatic |    | Human | Dose | C_{max} | AUC_{0-24h} | F% | T_{1/2} | V_{ss} | Comments |
| Nitazoxanide | Launched | CpPGR | NA | NA | NA | Yes in immunocompetent individuals | 100 mg single dose (oral suspension, children 1-3 years) | 3.11 \, \mu g/ml | 0.010 | NR | 1.03–1.6 h | NR | All reported parameters are based on the metabolite, tizoxanide, tizoxanide is highly protein bound (99%) |  |
| Clofazimine | Clinical | CpPMK | NA | NA | NA | Weekly active at 50 mg/kg (unpublished) | 100 mg Lamprene (day 5, part A and B in clinical trial) | 2807 nmol/ml (part A), 31.4 ng/ml (part B) | 66.5 mg/ml (part A), 11.2 mg/ml (part B) | 33.6 h (part A); 33.5 h (part B) | NR | Low bioavailability, high lipophilicity and permeability in C. hominis; clinical trial data showed about two-fold less plasma exposure in patients without diarrhea (part B); less than 2% of the cumulative CFZ doses was recovered in stool in 5 days of stool collection |
| KOU731 | Preclinical | CpP49K | 0.063 | 0.028 | 0.345 | Efficacious at 10 mg/kg q.d. | ND | 2.3 mg/kg (oral, mouse); 5 mg/kg (oral, calf) | 406 mg/ml (oral, mouse); 228 mg/ml (oral, calf) | 2306 mg/ml (oral, mouse); 75 mg/ml (oral, calf) | 6% (oral, mouse); 1.121 (oral, mouse) | NR | No correlation between efficacy and plasma exposure |
| BK-1708 | Predential | CpDMPK | 0.41 | NR | 0.0007 | Efficacious at 10 mg/kg q.d. | ND | 86.5–1070 mg/kg (oral, mouse) | 2.9 mg/ml | 267 ng/ml (oral, mouse) | 42.6 min | NR | Voiced PK/PD across three scaffold series scaffolds, GI exposure necessary for efficacy, no correlation with plasma exposure |
| AN1793 | Discovery (late lead) | CpPSF3 | 0.13 | 0.43 | 0.63 | Efficacious at 10 mg/kg q.d. | ND | 10 mg/kg (oral, mouse); 5 mg/kg (oral, calf) | 8.63 mg/ml (mouse); 3.37 mg/l (oral) | 92.7 mg/ml (mouse); 159 mg/ml (oral) | 37% (oral, mouse); 6.6 h | NR | Half life ~5 x greater in calves from none; high concentrations found in feces |
| Compound 2093 | Discovery (late lead) | CpMRS | 0.004 | 0.029 | 0.015 | Efficacious at 50 mg/kg b.i.d. | ND | 50 mg/kg (oral, mouse) | 5.4 mg/ml | 183 mg/min | ND | NR | C. hominis potency studies: plasma and fecal levels (3 x EC90 for over 24 h) |
| MM665917 | Discovery (late lead) | CpMRS | 1.9–2.3 | 4.1 | 0.06 | Efficacious at 30 mg/kg b.i.d. | ND | 55 mg/kg (oral, mouse); 22 mg/kg (oral, calf); 10 and 20 mg/kg (oral, piglet) | 5.4 mg/ml | 1340–1000 mg/min/ml (oral) | 100% | 5.5 h (v) | 4 mg/l | NR | PK in healthy mice; high fecal and plasma concentrations with sustained exposure; PK in infected cell model: sustained fecal and serum concentrations (3 x EC90)PK from infected-piglet model: plasma exposure remained (3 x EC90); guinea pig and Macaca nemestrina showed concentrations >EC90 60 h after treatment |
| Compound 5 | Discovery (late lead) | CpMRS | 1.3 | 6.0 | 0.13 | Efficacious at 20 mg/kg q.d. | ND | 10 mg/kg (oral, mouse); 3 mg/kg (oral, calf); 10 and 20 mg/kg (oral, piglet) | 5.4 mg/ml | 1300–1000 mg/min/ml (oral) | 100% | 2.5 h (v) | 11 Ag (v) | NR | Very high oral bioavailability |
| BIRD9299 | Discovery (late Op) | CpMRS | 0.008 | 0.073 | 0.10 | Efficacious at 10 mg/kg q.d. | ND | 1 mg/kg (oral, mouse); 0.6 mg/kg (oral, piglet) | 1 mg/kg (oral, mouse); 0.6 mg/kg (oral, piglet) | 100% | 80% | 11 Ag (v) | NR | BIRD9299 has high oral bioavailability, volume of distribution, and solubility, compounds in series with higher bioavailability tend better efficacy, possibly because of permeability |

AUC_{0-24h} area under the curve; time-averaged concentration of drug in plasma; C_{max}, maximum or peak serum (plasma) concentration of a drug after a single dose; F%, oral bioavailability; GB piglet, gnotobiotic piglet model; C. hominis; T_{1/2}, half-life in plasma; V_{ss}, volume of distribution at steady state.
suppressed oocyst shedding and diarrheal symptoms within the first 4 days postinfection, but then diarrhea and oocyst shedding rebounded in two of three animals. Subsequent sequencing of fecal samples revealed the acquisition of either a D243E or T246I mutation in C\textit{p}MetRS. Structural modeling indicated these mutations disrupted compound binding to C\textit{p}MetRS. In-vitro studies with recombinant enzymes containing these mutations were more than 170-fold less sensitive to inhibition by compound 2093, and \textit{C. parvum} parasites engineered with either mutation via CRISPR-Cas9 were found to be 613-fold (D243E) or 128-fold (T246I) less sensitive to compound 2093. These results demonstrate that resistance to C\textit{p}MetRS inhibition arose rapidly \textit{in vivo} and necessitate caution for future development and introduction of cryptosporidiosis therapeutics.

**Calcium-dependent protein kinase 1**

Calcium-dependent protein kinases (CDPKs) are an attractive drug target for apicomplexan diseases as they are essential and have no analogous proteins in mammals [17]. Bumped kinase inhibitors (BKIs) are ATP-competitive inhibitors of CDPKs and named for a structural bump that prevents binding in kinases with larger ‘gatekeeper’ residues in the ATP-binding pocket [18]. Researchers at the University of Washington discovered that a selection of \textit{Toxoplasma gondii} CDPK1 (TgCDPK1) BKI compounds also inhibit C\textit{p}CDPK1 [19]. Medicinal chemistry efforts over the past decade have resulted in several hundred BKI compounds displaying a range of pharmacokinetic/pharmacodynamic properties and antiparasitic activity [20], with the majority represented by three scaffolds: pyrrolopyrimidine (PrP), pyrazolopyrimidine, and 5-aminopyrazole-4-carboximide. Early leads showed good efficacy in mouse, calf, and gnotobiotic piglet models of cryptosporidiosis, though this series of compounds (subsequently referred to in this review as ‘series’) has faced challenges related to cardiovascular toxicity, teratogenicity, and varying efficacy because of the differing pharmacokinetic/pharmacodynamic parameters across scaffolds [21,22].

Due to ongoing toxicity liabilities in the pyrazolopyrimidine and PrP scaffolds [23], the most promising candidates have come from the 5-aminopyrazole-4-carboximide scaffold series. A recent report identified two 5-aminopyrazole-4-carboximide scaffold preclinical leads (BKI-1708 and BKI-1770) with good in-vitro potency against both the C\textit{p}CDPK1 enzyme and cells, minimal human \textit{Ether-a-go-go Related Gene} (hERG) activity, and good efficacy and safety in a mouse model of cryptosporidiosis (Table 1) [24]. Within the 5-aminopyrazole-4-carboximide scaffold series, high solubility but not high plasma or fecal exposure correlated with in-vivo efficacy. Both BKI-1708 and BKI-1770 show good in-vivo efficacy and alleviate many of the toxicity and safety liabilities of the BKI series, and warrant further study as preclinical candidates.

**Candidates from phenotypic screening efforts**

Another approach for finding new cryptosporidiosis therapeutics is phenotypic screening. Phenotypic screens are less biased and not dependent on a known target, and several emerging therapeutics for cryptosporidiosis were discovered from screening compound collections with known activity in other pathogens against \textit{Cryptosporidium} spp. [25]. Phenotypic screens may identify compounds with novel targets or pathways, with the caveat that target identification and MoA studies are often needed after active compounds are discovered. There have been a number of additional phenotypic screens for \textit{Cryptosporidium} drug discovery [25,26], though the compounds discussed in this review are the most advanced.

**Phosphatidylinositol 4-kinase 4 inhibitors**

Pyrazolopyridine inhibitors of Phosphatidylinositol 4-kinase 4 (PI(4)K) (a validated malaria drug target) were discovered from a phenotypic screen of 6220 parasite actives. Lead compound KDU731 was shown to have potent in-vitro activity against \textit{C. parvum} and \textit{C. hominis}, excellent efficacy in mouse and calf models of cryptosporidiosis (Table 1) and demonstrated safety in various in-vitro tests and a rat toxicology study [27]. Further in-vitro activity profiling showed that a related analog, KDU691, was parasiticidal at its EC\textsubscript{90}, and its MoA was impediment of merozoite formation [28], likely because of impairment of lipid membrane processing from inhibition of C\textit{p}PI(4)K. Interestingly, pharmacokinetics of KDU731 in \textit{C. parvum}-infected calves showed limited systemic exposure, indicating that it may not be required for clinical efficacy in this series.

**MMV665917**

A phenotypic high-content imaging screen of the Medicines for Malaria Venture (MMV) Malaria Box against \textit{C. parvum} conducted by researchers at the University of Vermont discovered MMV665917, a novel piperazine-based compound that is specific
Specific Factor 3 (CPSF3) based on the inhibition of the putative target is Cleavage and Polyadenylation Secrease, and was well tolerated in rodents and calves. Exposure, a long half-life (Table 1), high fecal exposure, and no significant organ toxicity was observed; however, MMV665917 partially inhibits hERG, indicating a potential for cardiac toxicity. It is possible that medicinal chemistry efforts can reduce the hERG inhibition potential of this scaffold while retaining the excellent anticryptosporidial activity, and the identification of the molecular target could greatly aid in these efforts.

AN7973

Another molecule that has emerged from a phenotypic screen of a focused collection of malaria active compounds is the benzoxaborole AN7973. Similar to MMV665917, AN7973 does not have a confirmed target or MoA but it has potent in-vitro activity against both C. parvum and C. hominis, and shows efficacy in both the IFNγ KO and NSG mouse models as well as the calf model of cryptosporidiosis [28,33]. In pharmacokinetic studies of AN7973 in mice and dairy calves, the compound displayed high plasma exposure, a long half-life (Table 1), high fecal exposure, and was well tolerated in rodents and calves. The putative target is Cleavage and Polyadenylation Specific Factor 3 (CPSF3) based on the inhibition of PfCPSF3 and TgCPSF3 by related benzoxaborole compounds and the shared catalytic core homology of this enzyme between these apicomplexan parasites [34], though more work is needed to confirm this as the target of AN7973 and related compounds in Cryptosporidium.

Pharmacokinetic/pharmacodynamic drivers of in-vivo efficacy

To understand the ideal pharmacokinetic profile for an anti-Cryptosporidium drug, researchers have explored various approaches including empirical observations of systemic and intestinal exposure, physiologically based pharmacokinetic modeling [35], and consideration of efflux pumps [36]. Although a broadly applicable profile remains elusive, in some cases, such as the BKIs and PI(4)K inhibitors, compound concentrations in intestinal epithelial cells seem to drive efficacy. In contrast, with PheRS inhibitors, systemic concentrations are most closely correlated with efficacy. Further work is needed to determine whether more broad observations can be made, or whether drivers are specific to individual targets, MoAs, or chemical series. Having clearly defined physiochemical and pharmacokinetic properties that drive in vivo and clinical efficacy for cryptosporidiosis therapeutics would help to prioritize emerging scaffolds and allow for efficient allocation of resources within drug discovery programs.

CLINICAL TRIAL OF CLOFAZIMINE AND LESSONS FOR FUTURE PROOF-OF-CONCEPT STUDIES

A repurposing candidate that emerged from a phenotypic screen, clofazimine (CFZ) [37], was quickly advanced into a clinical trial to assess its safety and efficacy in HIV-infected patients with cryptosporidiosis [38]. This phase 2a randomized, double-blind, placebo-controlled study had two parts: part A had primary outcomes of safety, pharmacokinetics, and reduction in Cryptosporidium oocyst shedding; part B was an open-labeled study comparing the pharmacokinetics of matched HIV-positive individuals without cryptosporidiosis (or diarrhea). The study faced many unexpected challenges with study initiation, population, implementation, and cultural issues [39**, though the sponsors were able to find solutions to successfully reach the endpoint. The study found treatment with CFZ had no significant impact on Cryptosporidium oocyst shedding, diarrheal episodes, stool weight, or consistency scores. Of note, the part A participants (with diarrhea) had about two-fold less plasma exposure of CFZ as compared with part B participants (no diarrhea), though no conclusions could be made whether this impacted the lack of efficacy. Although the results of the study do not support the efficacy of CFZ for treatment of cryptosporidiosis in a severely immunocompromised HIV population, this trial served to lay essential groundwork for future human studies to assess the efficacy of potential new anticyptosporidials [40].

In the past decade, increased awareness and funding toward finding a Cryptosporidium-specific therapeutic has resulted in a reasonably diverse pipeline; however, there is still a lack of a clinical proof of concept for a drug that is equivalent or superior to nitazoxanide. Specific use-case scenarios may also dictate the best clinical path for emerging therapeutics for cryptosporidiosis [12**]. Other practical considerations of treatment settings include...
the need for simple and affordable point-of-care diagnostics as well as reliable access to cryptosporidiosis treatments, that is, wherever children with diarrhea receive medical care and at nutritional rehabilitation and HIV treatment centers. These requirements are the most difficult to achieve in low-resource settings where the burden of Cryptosporidium infection is the highest. Accessible diagnostics and clearly defined diagnosis criteria are essential for deciding how to treat patients with new anticytosporidials [41], especially as there is a lack of guidance around empiric treatment or mass drug administration campaigns.

Another possible solution for deconvoluting the clinical path for emerging treatments for cryptosporidiosis could be a controlled human infection model (CHIM). It is feasible that the clinical pathway for anticytosporidial NCEs may diverge between the different target populations and use cases, and therefore, these studies could serve as a small-scale first pass to determine proof-of-concept efficacy of NCEs before a large financial investment in specific studies involving immunocompromised patients or young children. Although there is interest and precedence of CHIM for cryptosporidiosis [42], the most recent studies were conducted nearly two decades ago, and new regulatory requirements present unique challenges that complicate the re-establishment of this model [43].

To further complicate matters, the spontaneous resistance to CpMetRS inhibitor 2093 in the neonatal calf efficacy model [16] is extremely alarming and necessitates thoughtful design of future clinical trials and eventual implementation of new treatments. More studies are needed, ideally in early stages of development, to determine the frequency at which resistance mutations arise for this drug and others in the pipeline. Combination therapies and regimens are essential in combating other diseases with a large global health burden (e.g. malaria, HIV, tuberculosis), and the rollout of an effective therapy for cryptosporidiosis may quickly be rendered useless or exacerbate the burden of Cryptosporidium infection. Of note, there are currently no studies looking at any emerging therapeutics in combination.

CONCLUSION

The field of drug discovery for Cryptosporidium has made great strides in a short amount of time through both targeted approaches and phenotypic high-throughput screens; however, there is likely to be attrition of compounds as they progress through the later stages of development. The evolution of the clinical path for new anticytosporidial compounds must allow for advancement of promising compounds for key target populations and use-case scenarios while balancing the risks that may push away support from the pharmaceutical industry. The CRYPTOFAZ study was invaluable for lessons learned and capacity building efforts for future trials in resource-limited settings. Finally, implementation strategies of new drugs are critical. Even the best drug for treating Cryptosporidium infection will have limited impact if it cannot be effectively implemented with accessible and accurate diagnostics, and monotherapy strategies may be problematic if spontaneous resistance selection is observed with other compounds.

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Conflicts of interest

There are no conflicts of interest.

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• of special interest

•• of outstanding interest

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