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REVIEW

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Phenotypic screening techniques for *Cryptosporidium* drug discovery

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ABSTRACT

Introduction: Two landmark epidemiological studies identified *Cryptosporidium* spp. as a significant cause of diarrheal disease in pediatric populations in resource-limited countries. Notably, nitazoxanide is the only approved drug for treatment of cryptosporidiosis but shows limited efficacy. As a result, many drug discovery efforts have commenced to find improved treatments. The unique biology of *Cryptosporidium* presents challenges for traditional drug discovery methods, which has inspired new assay platforms to study parasite biology and drug screening.

Areas covered: The authors review historical advancements in phenotypic-based assays and techniques for *Cryptosporidium* drug discovery, as well as recent advances that will define future drug discovery. The reliance on phenotypic-based screens and repositioning of phenotypic hits from other pathogens has quickly created a robust pipeline of potential cryptosporidiosis therapeutics. The latest advances involve new *in vitro* culture methods for oocyst generation, continuous culturing capabilities, and more physiologically relevant assays for testing compounds.

Expert opinion: Previous phenotypic screening techniques have laid the groundwork for recent cryptosporidiosis drug discovery efforts. The resulting improved methodologies characterize compound activity, identify, and validate drug targets, and prioritize new compounds for drug development. The most recent improvements in phenotypic assays are poised to help advance compounds into clinical development.

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Challenges in *Cryptosporidium* drug discovery; cryptosporidiosis; *Cryptosporidium*; drug discovery; high-throughput screening; *in vitro* culture; phenotypic assay; phenotypic screening; pipeline

1. Introduction

Diarrheal disease continues to be a global health burden and a major cause of morbidity and mortality in children under the age of five, especially in resource-limited settings. Diarrhea, pneumonia, and malaria account for nearly a third of global deaths under the age of five [1]. *Cryptosporidium* spp. are a major cause of diarrheal disease in children ages 0–24 months, and are associated with an increased risk of death [2,3]. Young children who are immunocompromised, e.g., HIV-positive or malnourished, are highly susceptible to cryptosporidiosis. Additionally, immunocompromised adults (e.g., HIV-positive [4], organ transplant [5], or cancer patients [6]) are at risk of infection, though the disease is self-limiting in adults with healthy immune systems. *Cryptosporidium hominis* and *Cryptosporidium parvum* are the two main species that cause disease in humans, though there are roughly 40 recognized species with a wide range of hosts [7]. *C. parvum* is also an important cause of diarrheal and enteric disease in neonatal calves, which can have significant long-lasting impacts on the health of the animals and economic repercussions for farmers [8].

Cryptosporidium spp. are apicomplexan protozoan parasites with a complex life cycle that undergo both asexual and sexual reproduction in a single host. Spread via oral-fecal transmission, environmentally hardy thick-walled oocysts initiate infection after ingestion by the host, usually through contaminated food or water, or via direct contact with infected humans or animals. The primary site of infection is the small intestine, producing watery diarrhea and shedding of oocysts with the feces [9]. The single approved drug, nitazoxanide (NTZ; tradename Alinia®), has moderate efficacy in the most at-risk patients for cryptosporidiosis.

NTZ was first discovered in 1984 as a human cestocidal drug [10] and was later repurposed as a potential therapeutic for cryptosporidiosis, as there was no other effective drug for treating diarrhea caused by *Cryptosporidium* spp [11,12]. Clinical trials to assess the effectiveness of NTZ in multiple populations were pursued [13–15], and NTZ was approved in 2002 by the U.S. Food and Drug Administration (FDA) for the treatment of diarrhea caused by *Cryptosporidium* spp. and *Giardia intestinalis* in pediatric patients age 1–11, and for patients ≥12 years of age in 2005, although no benefit has been observed in HIV-positive patients with cryptosporidiosis and underscores efficacy is only observed for those without a compromised immune system [16].

Cryptosporidium was first described by E.E. Tyzzer in 1907 [17], but was not recognized as a significant cause of gastrointestinal disease in humans until much later in 1976 [18]. Since that time, cryptosporidiosis was known to be a cause of chronic diarrhea in AIDS patients and malnourished children and associated with waterborne and zoonotic outbreaks, but was largely under-recognized and underdiagnosed, both in the developed world and in resource-limited settings.

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Article highlights

- *Cryptosporidium* spp. were identified in 2013 as a significant cause of diarrheal disease in children 0–24 months in resource-limited countries, though the only drug for treatment is not effective and not indicated for children under 12 months. Since this revelation, a number of drug discovery programs have emerged to find new and more effective therapeutics.
- *Cryptosporidium* spp. have presented many challenges for studying and perturbing their biology. Using immortalized cell lines, four different phenotypic screening assays were established to enable high-throughput screens for drug discovery. However, the parasite does not complete its life cycle in this format, and more physiologically relevant culturing methods were needed to fully characterize compound activity.
- A diverse pipeline has been populated with potential candidates, the large majority of which were discovered via phenotypic screening and/or repositioning compounds from phenotypic screens against other neglected tropical diseases.
- Recent advances have been focused on the improvement of culturing capabilities to enable target identification, *in vitro* genetic modification, and phenotypic 'fingerprinting' to prioritize compounds in the preclinical development pipeline.
- Many great advances have been made in a short time period for *Cryptosporidium* drug discovery which are expected to help translate into clinical candidates for the treatment of cryptosporidiosis.

This box summarizes key points contained in the article.

Importantly, two seminal epidemiological studies published between 2013 and 2015 highlighted the true prevalence and burden of *Cryptosporidium* spp. in young children [2,19]. These studies were important in raising awareness to the prevalence of this disease and triggered a 'call-to-arms' to identify new and effective therapeutics to treat cryptosporidiosis [20].

Drug discovery efforts in the modern era typically begin with either a target-based or phenotypic-based approach. Target-based screening is a method of testing either small molecules, macromolecules, or biologics against a known target (e.g., an isolated enzyme or receptor). A distinct advantage of a target-based screen is the capitalization on knowledge around the target and/or its active site, which can result in a rational structure-based drug design strategy [21]. Target-based approaches also lend themselves to high-throughput screening (HTS), often are less resource-intensive and have reduced assay variation than cell-based methods. In contrast, phenotypic screening is an approach to discover molecules or compounds in a complex biological environment that exert a measurable effect on one or more aspects of the biological system, which can include both cell-based assays as well as animal models [22]. Although phenotypic screening generally requires a more challenging optimization process and resources, one main benefit to a phenotypic approach is less inherent bias because it does not rely on a specific target or pathway. Additionally, because the screen is conducted in a cell-based method, there is an added level of physiological relevance that an isolated target or purified protein lacks (e.g., confirmed cell permeability or uptake). A survey of new medicines over the ten-year period from 1998 to 2008 found that phenotypic approaches have had more translational success for the discovery of first-in-class drugs, whereas follow-on drugs are much more likely to have been discovered via target-based screening [21]. This was especially true for infectious diseases – seven new molecular entities were discovered via phenotypic screening and by contrast only three through target-based screens [21].

Prior to the publication of the genomic sequence for *C. parvum* and *C. hominis* in 2004, cryptosporidiosis drug discovery efforts had been scarce [23,24]. Naturally, the genomes of these species were mined for potential drug targets. Interestingly, compared to other apicomplexan parasites, *Cryptosporidium* has a pared down genome and mostly relies on salvage pathways as opposed to *de novo* biosynthesis for many of its nutrients. Despite the reduction in biosynthetic pathways, several targets were identified [25,26]; however, target validation using genetic methodologies was not a viable approach prior to the groundbreaking work by Boris Striepen and colleagues describing targeted genetic manipulation of *Cryptosporidium* via CRISPR/Cas9 [27].

Cryptosporidium also poses many additional challenges for drug discovery – as it is an obligate intracellular parasite, the host cell environment for *in vitro* assays can greatly affect the biology and therefore potential efficacy of compounds. *In vitro* assays of *Cryptosporidium* parasites are limited by three significant factors: (i) the ability to sustain a continuous culture, (ii) reliable cryopreservation methods, and (iii) the uncertainty of *in vivo* correlation. While the human ileocecal adenocarcinoma HCT-8 cell line has been used most frequently to culture and assay *Cryptosporidium* parasites, it is an imperfect system because *Cryptosporidium* spp. do not complete the sexual stages of the life cycle within this cell line and arrest before the formation of oocysts [28]. It has been observed that the parasite only undergoes two rounds of asexual replication within HCT-8 cells, allowing for a small window of time to examine drug effects before the parasite life cycle begins to shunt into sexual stage development and then prematurely arrests. Further, the absence of a reliable cryopreservation method is limiting because maintenance of strains of *Cryptosporidium* spp. is reliant upon continual passaging through animals (usually neonatal calves for *C. parvum*), and therefore sexual recombination and genetic drift are inevitable and unavoidable. Additionally, it has been difficult to predict *in vivo* efficacy from *in vitro* activity pharmacokinetic/pharmacodynamic parameters [29] and a clear relationship between animal models and clinical efficacy is as yet undetermined.

Here we review and discuss the status of phenotypic screening for drugs against *Cryptosporidium*, and how emerging technologies will improve and shape future capabilities. We will also review what drug candidates have emerged from previous and ongoing efforts, and discuss lessons learned and how they can be applied to any new efforts to find effective therapeutics for cryptosporidiosis.

2. Screening strategies for cryptosporidiosis drug discovery

In the years following NTZ's approval, multiple strategies have been employed to find new druggable targets and active

Table 1. Pipeline of potential cryptosporidiosis therapeutics.

Representative Compound	Phase of Development	Discovery Method	Source Library	MOA/Target	*In vitro* activity (EC_{50}, μM)	*In vivo* activity
Nitazoxanide [12,30]	Approved – Standard of Care	Repurposed	N/A	Inhibits *Cp*PFOR, possibly others	*Cp* and *Ch*: ~3	Ineffective in animal models of cryptosporidiosis
Clofazimine [67,69]	Clinical – Ph2a	Phenotypic (HCI)	10,000 set of Bioactives	Unknown	*Cp* HCI: 0.015 *Ch* HCI: 0.340	Efficacious at 10 mg/kg QD in IFNγ KO mouse model; Not efficacious in NSG mouse model; Clinical trial did not show efficacy in HIV+ adults with cryptosporidiosis
KDU731 [78]	Preclinical – PCC	Repositioned: Phenotypic (HCI, *Cp*Luc, CPE)	6,220 focused set with known antiparasitic activity	*Cp*PI(4)K	*Cp* CPE: 0.107 ± 0.039 *Cp* HCI: 0.063 ± 0.028 *Ch* CPE: 0.130 ± 0.074	Efficacious at 10 mg/kg QD in IFNγ KO mouse model; Efficacious at 5 mg/kg BID in neonatal calf model
BKI-1369 [86,90]	Preclinical – PCC	Repositioned: Target-based (qRT-PCR, *Cp*Luc)	CDPK1-focused library; originally developed for *T. gondii*	*Cp*CDPK1 inhibitors, possibly others	*Cp* Luc: 0.344	Efficacious at 30 mg/kg BID in IFNγ KO mouse model; efficacious at 10 mg/kg BID in calf and piglet (*Ch*) models
AN7973 [83]	Discovery – Late lead	Repositioned: Phenotypic (HCI)	Focused library of 7,802 compounds from other NTDs	Unknown, possibly *Cp*CPSF3	*Cp* HCI: 0.13–0.43 *Ch* HCI: 0.52	Efficacious at 10 mg/kg QD in IFNγ KO and NSG mouse models and calf model
VB-201 [31]	Discovery – Late lead	Phenotypic (HCI)	12,000 known drugs: ReFRAME library	Unknown; VB-201 has been shown to inhibit TLR-2/4 in humans	*Cp* HCI: 0.860	Efficacious at 10 mg/kg BID in IFNγ KO mouse model
Vorinostat [74]	Discovery – Late lead	Phenotypic (qRT-PCR)	1,200 known drugs from the Prestwick library	*Cp*HDAC	*Cp* qRT-PCR: 0.203	Efficacious at 10 mg/kg QD in IL-12 KO mouse model
Compound 2093 [96]	Discovery – Lead Op	Repositioned: Target-based (biochemical)	Focused library of <500 compounds from antitrypanosomal and antibacterial MetRS inhibitor programs	*Cp*MetRS	*Cp* HCI: 0.007 *Cp* Luc: 0.036	Efficacious at 50 mg/kg BID in IFN γ KO and NSG mouse models
MMV665917 [32,33,47]	Discovery – Lead Op	Phenotypic (HCI)	400 compounds from the MMV Open Access Malaria Box	Unknown	*Cp* HCI: 1.9–2.3 *Ch* HCI: 4.05	Efficacious at 30 mg/kg BID in IFNγ KO mouse model; efficacious of 60 mg/kg BID in NSG mouse model; efficacious at 22 mg/kg QD in calf model; efficacious at 20 mg/kg BID in piglet model
Compound 5 [95]	Discovery – Lead Op	Phenotypic for malaria; Repositioned: Target-based for *Cryptosporidium*	GSK Tres Cantos Antimalarial Set ~13,000 compounds; repositioned from malaria KRS program	*Cp*KRS inhibitor	*Cp* Luc: 2.5 *Cp* HCI: 1.3 *Ch* HCI: 6.0	Efficacious at 20 mg/kg QD in IFNγ KO and NSG mouse models
BRD7929 [94,97,126]	Discovery – Lead Op	Phenotypic for malaria; Repositioned: Target-based for *Cryptosporidium*	Broad DOS library of 100,000 compounds; Repositioned from malaria PheRS program	Antimalarial compound targeting *Pf*PheRS; putatively targeting *Cp*PheRS	*Cp* qRT-PCR: 0.033	Efficacy at 10 mg/kg in NSG mouse model

(*Continued*)

Table 1. (Continued).

Representative Compound	Phase of Development	Discovery Method	Source Library	MOA/Target	*In vitro* activity (EC_{50}, μM)	*In vivo* activity
P131 [34,35,99–101]	Discovery – Lead Op	Target-based (biochemical)	44,522 compounds from multiple libraries	*Cp*IMDPH	*Cp* HCI: 7	Efficacious at 250 mg/kg once, or 83 mg/kg QD for 3 days in IL-12 KO mouse model
Triacsin C; R134 [104,105]	Early Discovery	Target-based (biochemical); Virtual *in silico* screen	515 Triacsin C + analogs	*Cp*LC-FACS, isoforms *Cp*ACS1 and *Cp*ACS2	*Cp* qRT-PCR: 0.136	Efficacious at 15 mg/kg QD in IL-12 KO mouse model
Itavastatin [62]	Early Discovery	Phenotypic (HCI)	727 FDA-approved drugs or drug-like compounds from the NIH Clinical Collections libraries	*Hs*HMG-CoA Reductase, may lead to low levels of host IPP (building block for *Cryptosporidium* isoprenoids)	*Cp* HCI: 0.62	Not Tested
4 compounds [103]	Early Discovery	Target-based (biochemical)	1,040 compounds from Microsource Discovery Systems and NIH/JRDF custom collection	*Cp*ACBP1	*Cp* qRT-PCR: range 25.1–64.9	Not Tested
16 compounds [36]	Early Discovery	Phenotypic (qRT-PCR)	NatProd Collection – 800 compounds from natural products	Unknown	*Cp* qRT-PCR: range 0.122–3.940	Not Tested
Herbicidins (5 compounds) [70]	Early Discovery	Phenotypic (HCI)	3,127 fractions prepared from a collection of 159 microbial strains	Unknown	*Cp* HCI: range 0.15–24	Not Tested

compounds for *Cryptosporidium* spp., creating a pipeline of potential cryptosporidiosis therapeutics Table 1. The current pipeline is the culmination of a steady increase in drug discovery efforts for cryptosporidiosis, a trend likely to continue as increased disease awareness, additional funding sources and new technologies will enable improved assay platforms to investigate and exploit parasite biology. Table 1 highlights nearly twenty candidates in various drug discovery stages ranging from early discovery to Phase 2a human proof-of-concept studies. Overwhelmingly, phenotypic assays were used to identify these candidates or hit series, reflecting the preferential screening approach for neglected tropical diseases and reinforcing the limited number of validated *Cryptosporidium* drug targets for biochemical assay screens.

A successful drug discovery program for small-molecule inhibitors must first identify compounds that exert the desired effect (on a drug target, a cell model, or an animal model; Figure 1), and then extensive profiling is needed to determine specificity, selectivity, and safety. While this review has a primary focus on the *in vitro* phenotypic assays to support cryptosporidiosis drug discovery, animal models of cryptosporidiosis also represent a key phenotypic model to validate compound efficacy in the drug discovery process.

In brief, there are several immunocompromised animal models to study *C. parvum* and *C. hominis*, as well as the neonatal calf challenge model. The only experimental animal model of *C. hominis* to assess potential therapeutics is the gnotobiotic piglet model [37,38], which is difficult, expensive, and specific to a single laboratory. Because *C. parvum* naturally infects young calves, producing acute and fulminant diarrhea, this model is thought to possibly be the most clinically relevant. The primary limitations of the neonatal calf model are that specialized facilities and large quantities of experimental compound (~5–15 g) are required to support this model [39–41]. Thus, rodent models are more attractive for their sheer convenience and significantly reduced compound requirements (~20–100 mg per experiment). Immunocompromised mice have been recognized as sufficient hosts to support *C. parvum* infections. Host control and resolution of *Cryptosporidium* infection require interferon gamma (IFNγ) [42,43], and interleukin-12 (IL-12) can induce IFNγ production, therefore experimental mouse models have been established in IFNγ knockout (KO) mice [44], IL-12 KO mice [45,46], and severely immunocompromised NOD scid gamma (NSG) mice [47] as well as others [48]. Importantly, the translation of these animal models to human cryptosporidiosis have not been established and a further conundrum is how well the activity observed for *in vitro* assays correlate to these *in vivo* models. Thus, the continued development and incorporation of predictive *in vitro* assays have been a primary consideration for those initiating drug discovery programs for cryptosporidiosis.

Although the methods of discovering candidate drug molecules can vary, the most common approach is to screen against a known target or conduct an unbiased phenotypic screen against small-molecule compound libraries. As the number of drug discovery efforts for the treatment of cryptosporidiosis have increased in recent years, so too have the available screening formats. Here, we review the major anti-cryptosporidial drug discovery efforts and categorize the approach according to the primary assay: 1) phenotypic-based screen, 2) the classical target-based screen, or 3) a hybrid approach that focuses on repositioning compounds or drug targets derived from phenotypic screens against other pathogens.

2.1. Phenotypic-based assays for *Cryptosporidium* drug discovery

In vitro cultivation of *Cryptosporidium parvum* for the purpose of screening for anti-cryptosporidial compounds was first

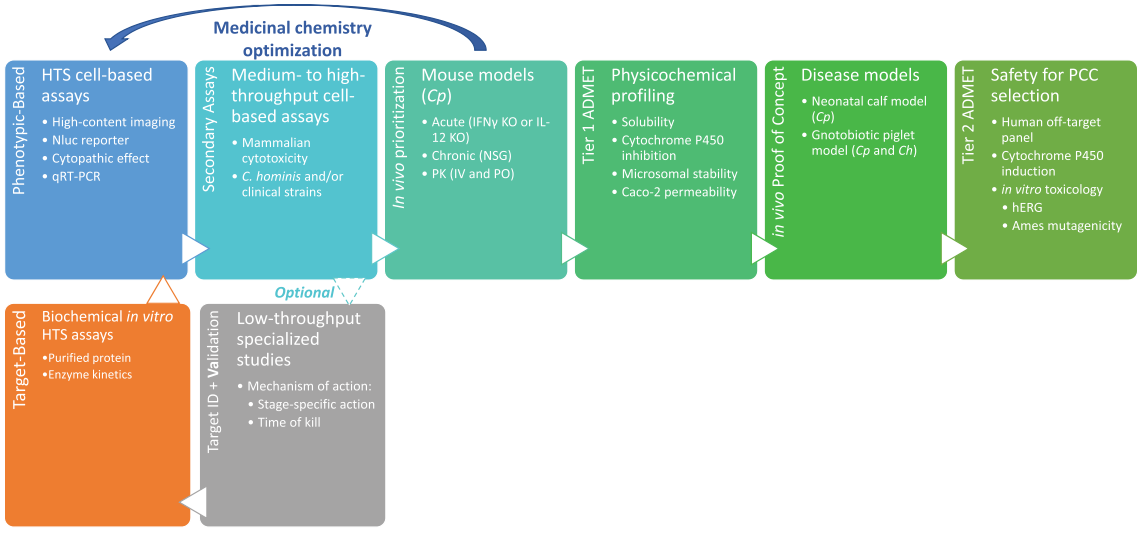

Figure 1. **Flowchart of proposed preclinical *Cryptosporidium* drug discovery workflow**. The top panels typify the more popular approach with phenotypic *in vitro* assays and mouse *in vivo* models to support the discovery and medicinal chemistry optimization of anti-cryptosporidial compounds. Target-based approaches have also been incorporated in several efforts, but hits from target-based screens must be tested in phenotypic cell-based assays to confirm activity. Recent advances include lower-throughput assays for target identification and examination of molecular mechanism of action. These specialized assays are currently optional and orthogonal to the workflow but are likely to become essential as pipeline prioritization is needed. ADMET is absorption, distribution, metabolism, excretion, and toxicity; hERG is the human ether-à-go-go-related gene; PCC is a pre-clinical candidate.

Table 2. Phenotypic screening assay formats for *Cryptosporidium* spp.

Assay type	Short	*Cryptosporidium* species supported	Advantages	Disadvantages
High-content imaging [62,66]	HCI	*C. parvum; C. hominis*	Allows for concomitant readout of both host cell and *Cryptosporidium* growth inhibition; fixation allows for flexible analysis; miniaturized to 1536-well format	Requires many steps for fixation and staining; requires special imaging equipment
Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction [72,73]	qRT-PCR	*C. parvum*	Use of whole well for lysates reduces counting errors from dead parasites; allows for assessment of host cell quantification	96-well assay format limits throughput; requires specialized instrumentation; high cost of reagents
Cytopathic Effect [77]	CPE	*C. parvum; C. hominis*	Simple one-step addition of commercially available detection reagent; allows for selection of non-cytotoxic compounds; 24 h post-infection compound addition	Requires large quantity of oocysts (high MOI); direct parasitic activity must be confirmed in a secondary assay format
Luciferase-expressing reporter strain [27]	*Cp*Luc	Nanoluciferase-expressing *C. parvum*	Simple one-step addition of commercially available detection reagent; luminescence readout is robust, stable, and quick	Restricted to genetically modified *C. parvum*

described in 1990 [49]. Multiple studies improved upon this strategy by evaluating myriad different host cells to culture *Cryptosporidium* parasites [50–56], with HCT-8 cells being used most frequently today. While this enabled a few relatively small (<1,000 compounds) screening efforts [57–61], the lack of continuous culture and preservation methods left a large dearth of phenotypic screening efforts for *Cryptosporidium*. Four cell-based phenotypic assays for assessing drug activity against *Cryptosporidium* have been developed and used in many cryptosporidiosis drug discovery programs which we will review here (Table 2).

The first cell-based high-throughput screening assay for *Cryptosporidium parvum* was reported in 2013 by Bessoff *et al*. [62]. The authors built upon previous techniques and married them together to create a 384-well imaging-based assay. In brief, *C. parvum* oocysts were excysted and added to HCT-8 cells and treated over a 48-hour period before being fixed and imaged to determine parasite growth inhibition. Cell-based imaging assays often rely on antibodies for labeling specific cell types or features, but commercially available antibodies against *Cryptosporidium* have been severely limited; the authors of this study creatively took advantage of a lectin that binds to the N-acetyl-D-glucosamine (GlcNAc) present on the surface of *Cryptosporidium* and selectively labels the parasites but not the host cells [63,64]. Though not trivial and requiring specialized imaging equipment, microplate washing and dispensing capabilities, the assay is reproducible and allows for the concomitant assessment of both parasite and host cell growth. The authors developed this assay to screen the NIH Clinical Collections (727 FDA-approved drugs or drug-like compounds) and the MMV Open Access Malaria Box (400 compounds), which namely resulted in the identification of itavastatin, a cholesterol-lowering medication that inhibits 3-hydroxy-3-methyl-glutaryl-CoA reductase, a rate-limiting enzyme in the mevalonate pathway, and MMV665917, an antimalarial compound with unknown mechanism of action [47,65].

The high-content imaging (HCI) assay from Bessoff *et al*. was further miniaturized and optimized to allow for higher throughput and screening of larger compound libraries, and also adapted to testing *C. hominis* for *in vitro* activity as a secondary assay [66]. A screen of 78,942 compounds comprised of 10,000 known Bioactives and 68,000 small molecules from the Global Health Chemical Diversity Library was performed which led to the discovery of clofazimine (CFZ), a riminophenazine dye that is indicated for multibacillary leprosy and has known activity against *Mycobacterium tuberculosis* [67]. Excitingly, because of its long history of clinical use, safety profile, activity in the IFNγ KO mouse model of cryptosporidiosis, and low cost of goods, CFZ was advanced to a Phase 2a proof-of-concept clinical trial for the treatment of cryptosporidiosis in HIV-positive adults [68]. Unfortunately, the findings of the trial do not support CFZ for the treatment of cryptosporidiosis in severely immunocompromised populations, but the trial marked the first time a molecule from a screening campaign for cryptosporidiosis was advanced into the clinic [69]. Other potential compounds discovered from HTS campaigns with this HCI assay include TLR2/4 inhibitor oxidized phospholipid VB-201 and TrkA receptor antagonist ASP-7962 from the Repurposing, Focused Rescue, and Accelerated Medchem (ReFRAME) library, a drug repositioning collection of ~12,000 compounds (since expanded to >14,000). A small screen of fractions of natural products isolated from 159 microbial strains was performed as well, identifying five herbicidins with potent *in vitro* activity against *C. parvum* [70].

A second method for assessing *Cryptosporidium* growth *in vitro* is through quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) [71]. This assay format was initially reported in 2005 and then later adapted to an HTS 384-well format with the advent of commercial reagents that simplified the preparation of cell lysates suitable for qRT-PCR analysis [72,73]. This assay has been used to screen 1,200 compounds from another library of known drugs – the Prestwick Chemical Library – which has resulted in multiple compounds of interest with novel targets and mechanisms for *Cryptosporidium*, including the HDAC inhibitor vorinostat and several others [74–76]. Another screen of 800 natural products

with defined chemical structures was performed with this assay format, resulting in 16 with half-maximal effective concentration (EC_{50}) values <5 μM.

A third type of phenotypic assay is the cytopathic effect (CPE) assay – a method commonly used to assess inhibitors of viral replication. This assay was adapted to *Cryptosporidium* infection and optimized to a multiplicity of infection (MOI) in HCT-8 cells that gave a reproducible CPE to allow for 48-hour drug treatment [77]. Unlike the HCI- and qRT-PCR-based assays, the CPE assay indirectly measures parasite growth by directly measuring host cell survival, achieved through a commercial viability reagent such as CellTiter-Glo®. This assay has been adapted for both *C. parvum* and *C. hominis* infection and was validated by screening compounds with known anti-cryptosporidial activity [78]. The CPE assay is amenable to HTS, robust, easy, and also allows for filtering out cytotoxic compounds.

The fourth reported phenotypic assay for screening against *Cryptosporidium* came after the advent of genetic modification via CRISPR/Cas9 to generate luciferase-expressing reporter strains [27]. While this assay is robust and is now routinely used to support HTS as well as smaller focused activities [79,80], it is contingent upon access to specialized strains of *C. parvum*, which can prove difficult with no reliable cryopreservation. Both blue-shifted Nanoluciferase (Nluc) and red-shifted firefly luciferase (FFluc) expressing strains have been generated, though the Nluc line is preferred for *in vitro* assaying due to its high stability, ATP independence, and robust and high sensitivity. An additional benefit is that the commercially available Nano-Glo® can be used for reliable assay readout.

2.2. Repositioning phenotypic hits or drug targets from NTDs

A successful strategy employed amongst cryptosporidiosis drug discovery programs was to expedite screening efforts by repositioning phenotypic hit compounds or lead series from other pathogens. Specifically, *Plasmodium falciparum*, which is the causative agent of human malaria and is a distantly related apicomplexan parasite to *Cryptosporidium*, was a logical species to evaluate compound cross-reactivity. Although other hits emanating from screens against another apicomplexan human pathogen, *Toxoplasma gondii*, as well as kinetoplastid parasites have proven fruitful as well. In this section, we will focus on strategies that relied on existing knowledge and chemical matter to quickly find compounds that have activity against *Cryptosporidium* spp.

2.2.1. Repositioning of phenotypic screen hits

The Novartis Institute for Tropical Diseases (NITD) has a long history of drug discovery programs for numerous infectious diseases [81], including cryptosporidiosis. The NITD parasite box, a collection of 6,220 focused diversity set was screened against *C. parvum* with the HCI assay, resulting in the discovery of *Cryptosporidium* phosphatidylinositol-4-OH kinase (PI(4)K) inhibitor KDU731 as a potent inhibitor of parasite growth *in vitro* and *in vivo* in the IFNγ KO mouse and neonatal calf models of cryptosporidiosis [78]. The related kinase in *P. falciparum, Pf*PI(4)K, is a validated drug target for malaria [82], and the potent and selective anti-cryptosporidial activity of KDU731 suggests that this compound series should be further evaluated as a potential therapeutic for cryptosporidiosis.

Another promising lead discovered through a repositioning strategy is the benzoxaborole AN7973, originally from a collection of existing compounds from Anacor's (now a part of Pfizer) Neglected Tropical Disease portfolio – including antimalarial, antikinetoplastid, and an antibacterial leucyl-tRNA synthetase inhibitor program [83]. Though many of the specific targets of all compounds screened were unknown, the researchers used the HCI HTS assay to find potent inhibitors of *C. parvum* growth. The lead compound, AN7973, was identified with no further medicinal chemistry efforts for *C. parvum* and was found to have favorable physiochemical and pharmacokinetic properties that supported *in vivo* testing. Excitingly, AN7973 is highly efficacious in the IFNγ KO mouse, the NOD scid gamma (NSG) mouse, and neonatal calf models of cryptosporidiosis.

2.2.2. Repositioning validated parasite drug targets

Similar to other drug discovery efforts, the 'post-genomic' era of *Cryptosporidium* drug discovery saw a boom of target-based approaches with mixed success. *Cryptosporidium* has a small (~9 Mb) adenosine- and thymidine-rich (~70%) genome with a fairly large proportion of genes acquired through horizontal gene transfers [84,85], and has numerous transporters but few enzymes [25]. Attempting to find new anti-cryptosporidial compounds via a target-based approach without the guaranteed ability to validate the target afterward has greatly limited the number of these programs. Therefore, most target-based screens for *Cryptosporidium* drug discovery have been repositioned from other programs from related pathogens (e.g., *Plasmodium*). This target-biased approach takes advantage of both phenotypic- and target-based approaches by capitalizing on some prior knowledge around the target (even if unvalidated) while still casting a wide net.

One such target, *C. parvum* calcium-dependent protein kinase 1 (*Cp*CDPK1) was investigated as a potential drug target due to its similarity to the related CDPK1 from the apicomplexan *Toxoplasma gondii* (*Tg*CDPK1) [86]. Several potent inhibitors of *Cp*CDK1 were identified in an enzymatic assay, and then shown to block invasion of *C. parvum* into HCT-8 cells [86]. The role of *Cp*CDK1 was not fully understood initially, but the importance of calcium signaling in *C. parvum* was known and the shared sequence identity of both CDPK1 proteins was high (70%) [87]. These apicomplexan CDPK1 proteins have a unique glycine 'gate-keeper' residue that confers selectivity over human kinases [88,89]; the inhibitors are termed 'bumped kinase inhibitors,' or BKIs, as they do not bind in the human ATP-binding domain. Lead optimization strategies on BKIs over the past decade have resulted in preclinical leads from three BKI scaffolds with *in vivo* efficacy, but varying safety and tolerability [90–93].

Other leads discovered through repositioning include three that target (or are believed to target) *Cryptosporidium* aminoacyl-tRNA synthetases (aaRSs) – two from antimalarial programs [94,95], and one from an antikinetoplastid program [96]. BRD7929 is a bicyclic azetidine discovered from a phenotypic

cell-based malaria screen of 100,000 compounds from a library built via diversity-oriented synthesis (DOS); post-screening target identification studies have determined it is an inhibitor of *P. falciparum* phenylalanyl-tRNA synthetase (*Pf*PheRS). BRD7929 is a putative inhibitor of *Cp*PheRS, and as expected of an aaRS, shows potent activity against all life stages of *P. falciparum* and *C. parvum in vitro* and efficacy in mouse models of malaria and cryptosporidiosis [97]. A separate biochemical, target-based screen of another malaria aaRS – *Pf* lysyl-tRNA synthetase (*Pf*KRS) – was undertaken due to the previously identified cladosporin, which targets *Pf*KRS but is not suitable for progression into animal studies. The Tres Cantos Antimalarial Set from GlaxoSmithKline (GSK; ~13,000 compounds) was screened using recombinant *Pf*KRS via Kinase-Glo®, which identified a hit chromone (isomer of coumarin analog) Compound 2. The screen hit was further optimized for biological activity and pharmacokinetic profile to the lead Compound 5. The researchers characterized *Pf*KRS and *Cp*KRS, which have a 96% active site sequence identity, and found that this compound series retained activity against recombinant *Cp*KRS, as well as cultured *C. parvum* (Nluc-based assay) and *C. hominis* (HCI assay). The lead Compound 5 was advanced to *in vivo* studies and was efficacious in a mouse malaria model, as well as both the IFNγ KO and NSG mouse models of cryptosporidiosis. The third aaRS repositioning candidate – imidazopyridine Compound 2093 – came from screening a focused library (<500 compounds) of methionyl-tRNA synthetase (MetRS) inhibitors developed from a program to optimize their antitrypanosomal and antibacterial activities. These compounds were screened against *Cp*MetRS, and potent and selective inhibitors were identified. Hit compounds were prioritized based on pharmacological, physiochemical properties, and toxicology studies, resulting in several leads for further development.

These leads discovered through repositioning efforts show much promise and have progressed in the anti-cryptosporidial pipeline very quickly because medicinal chemistry optimization efforts were done prior to these efforts. In many cases, these compounds already had good pharmacological and physiochemical profiles that support further development before they were investigated for cryptosporidiosis. This target-biased approach allows for rational phenotypic or target-based screening while saving valuable time and resources.

2.3. Classical target-based screens

Although purely target-based efforts have been the less-commonly used method for finding new anti-cryptosporidials and are not the focus of this review, they are worth a brief examination. Table 1 highlights a number of compounds or series in the cryptosporidiosis pipeline and their breakdown by discovery method. The large majority of drug candidates (14/17) have been discovered through phenotypic or repositioning approaches, with the remaining 3 coming from purely target-based approaches. These efforts have also relied on phenotypic assays to confirm cell-based activity of hit compounds.

Cryptosporidium relies on inosine-5′-monophosphate dehydrogenase (IMDPH) for biosynthesis of guanine nucleotides, and was previously identified as a potential drug target for *Cryptosporidium* spp [98]. A biochemical target-based screen of >44,000 compounds was conducted against *Cp*IMDPH, which identified a number of hits [99] that were then carried into a program to develop these as potential treatments through medicinal chemistry [100–102]. These efforts have produced compound P131 which shows efficacy in the IL-12 KO mouse model of cryptosporidiosis. The original screen was published in 2008, and the latest studies include a co-crystal structure with P131 in 2015, but further efforts with this series have not been reported.

The Zhu lab at Texas A&M University has conducted a number of biochemical target-based screens for *Cryptosporidium* drug discovery in addition to developing the qRT-PCR phenotypic assay. One screen looked at a 1,040-compound set containing mostly known drugs against the sole *C. parvum* fatty acyl-CoA binding protein (*Cp*ACBP1) which resulted in four compounds with anti-cryptosporidial activity *in vitro* [103]. Another study used a known acyl-coenzyme A synthetase (ACS) inhibitor, Triacsin C, to explore *C. parvum* ACSs as potential drug targets by examining their effects in enzymatic assays, cell-based assays, and *in vivo* in the IL-12 KO mouse model [104]. This work was later expanded on recently with a virtual screen of 514 Triacsin C analogs against *C. parvum* long-chain fatty acyl coenzyme A synthetase (LC-FACS), resulting in a proposed compound (R134) as a putative drug candidate for *Cp*LC-FACS [105].

3. Enhanced *in vitro* assays with increased predictive qualities for compound profiling and prioritization

As the field of drug discovery for cryptosporidiosis continues to grow and evolve, so do the technologies and capabilities. The past five years have resulted in significant advances for high-throughput screens as well as the development of lower-throughput specialized assays to perturb parasite biology and characterize compounds. Previous advances, including *in vitro* culturing platforms that allowed for HTS for *Cryptosporidium* spp. enabled the discovery of many new and promising anti-cryptosporidial therapeutics, but the usual attrition rates of compounds as they progress through non-clinical and clinical studies necessitate the ability to prioritize additional differentiated compounds in the drug discovery pipeline. Paramount to the continued evolution of this field is the ability to recapitulate the entirety of the parasite life cycle *in vitro*. Such advancements support progress toward *in vitro* genetic manipulation methodologies and target identification studies. In this section, we explore new technologies that enhance *in vitro* culturing methods, and techniques for characterizing compounds to distinguish them by their molecular mechanism of action (MMOA).

3.1. Advances in *in vitro* culturing methods

Although many cell-based systems can support *Cryptosporidium* growth enough to assess gross inhibitory effects from drug treatment, many HTS assays have relied upon the use of transformed or immortalized host cells and therefore may have missed key pathways or factors in host-

pathogen interactions important in pathogenesis. Additionally, many cell culture systems for HTS do not support completion of the life cycle or extended propagation of *Cryptosporidium in vitro*. Recent studies have explored new types of host cells, including organoids or *ex vivo* explants, and modifications to the culturing environment to mimic the gut epithelium more closely, allowing for new techniques to probe the biology with potential drugs and tool compounds. Cell-free systems that display completion of the life cycle have also been explored [106,107], though these have not been widely adopted and lack the ability to explore the interplay between host and pathogen. Here we review the most impactful advances that will likely enable new approaches to phenotypic screening and drug discovery for cryptosporidiosis Table 3.

Morada *et al*. used hollow fiber technology to create a biphasic gut-like environment to culture *C. parvum*-infected HCT-8 cells [109]. The design of the hollow-fiber cartridge creates a surface for polarized host-cell growth with a basal aerobic nutrient supply, as well as an anaerobic compartment more suitable for *C. parvum* propagation. The authors were able to observe all developmental stages via immunofluorescent staining and obtain large numbers of infective *C. parvum* oocysts (10^8 per milliliter) distinct from seeded oocysts as far out as 18 months [110]. The potential manipulations of the environment and cultures allow for pharmacokinetic (PK) and pharmacodynamic (PD) explorations [111], though this method is cumbersome, expensive, and not easily scalable for multiple drugs and replicates.

DeCicco RePass *et al*. developed a three-dimensional (3D) bioengineered human intestinal tissue system with silk cocoons as the scaffold [112], creating a gut-like environment with a center lumen and allowing for polarized growth of host cells. Human intestinal myofibroblasts are grown within the silk material while Caco-2 and goblet cell-like methotrexate-differentiated HT29 (HT29-MTX) cells are seeded into the hollow center of the cocoon and then infected with *C. parvum* oocysts or purified sporozoites. Similar to the hollow fiber method, the authors were able to observe completion of the life cycle and oocyst formation and stably maintain the cultures for at least 8 weeks, likely longer (8 weeks were the longest timepoint tested). This method is on a much smaller scale than the hollow fiber cartridges and cannot produce large quantities of oocysts, but it may be more amenable to moderate-throughput screening or *in vitro* propagation of transgenic *C. parvum* lines. The authors have also adapted these 3D silk scaffolds into a multifunctional bioreactor to create a dynamic culture environment, allowing for control of oxygen levels in perfusion fluids, as well as the mechanical and chemical microenvironments present in human intestines [113]. The bioreactor system may be a much more physiologically relevant environment in which to study co-cultures of human pathogens such as *Cryptosporidium*.

Another simplified *in vitro* culturing system that allows for continuous propagation of *Cryptosporidium* parasites is the esophageal squamous-cell carcinoma cell line COLO-680 N [114]. The authors observed completion of the life cycle via

Table 3. Summary of advancements in *Cryptosporidium* culturing and phenotypic assays.

Platform	Cell Type	Application	Advantages	Disadvantages
Hollow fiber [108–110]	Human ileocecal adenocarcinoma cell line HCT-8	Continuous culture; *in vitro* propagation of oocysts; PK/PD studies	*C. parvum* generates large numbers of oocysts; Continuous cultures achieved for at least 18 months	Requires consistent maintenance, costly, cumbersome; not easily scalable, though smaller cartridges may enable replicates
3D silk scaffolds [112,113]	Human intestinal myofibroblasts; human IEC lines (Caco-2/HT29-MTX) as host cells	Continuous culture; *in vitro* propagation of oocysts; co-culture studies	Scalable for multiple drugs or replicates; amenable to a bioreactor system for mechanical and chemical microenvironments	Does not produce large number of oocysts; relies on transformed carcinoma-derived cell lines
COLO-680 N cells [114,115]	Human esophageal squamous-cell carcinoma cell line COLO-680 N	Continuous culture; *in vitro* propagation of oocysts	Simple 2D culture; *C. parvum* generates moderate numbers of oocysts; continuous culture for 4 months in a bioreactor system; cryopreservation of infected cells	Relies on transformed carcinoma-derived cell lines
Primary cells [116,117]	Primary human and/or bovine IECs	Small-scale biological studies in a physiologically relevant environment	Recapitulates intestine; supports *C. parvum* and *C. hominis* (human IECs); not reliant upon carcinoma-derived cell lines	Difficult to obtain; not easily scalable for high-throughput assays; do not support continuous culture
Organoids [118–121]	*Ex vivo* tissues or IECs	Continuous culture; *in vitro* propagation of oocysts; Small-scale biological studies in a physiologically relevant environment	Recapitulates intestine; not reliant on carcinoma-derived cell lines; unlike primary cells, allows for regeneration and continuous growth	Infection by *C. parvum* may inhibit propagation of organoids; parasite and oocysts counts decreased over time
Stem-cell derived IECs under ALI conditions [122,127]	Irradiated 3T3 feeder cells with spheroids derived from mouse IECs as host cells	Continuous culture; *in vitro* propagation of oocysts; co-culture studies	Transwell microtiter plates allow for simple drug treatment and washout, and is easily scalable for moderate throughput screening and/or multiple conditions	While accessible to most laboratory formats, specific reagents and skills are necessary; not amenable to imaging
Imaging-based phenotypic assays [97,126]	HCT-8 cells	Examine specific life stage effects of compounds; "Fingerprinting" compounds to study MMOA	Simple 2D culture; imaging allows for examine specific life stage effects	HCT-8 cells do not allow for completion of life cycle; relies on transformed carcinoma-derived cell lines

immunofluorescent staining, extended propagation out to at least 60 days post infection, and a large production of oocysts (50-fold increase compared to seed numbers after 10 days in culture; 120-fold after 60 days; 10^7 oocysts per milliliter). Although these cells may lack relevance compared to primary human intestinal epithelial cells, this method is simple, sustainable, and amenable to a standard laboratory, which offers great advantages over other two-dimensional (2D) immortalized cell lines for the propagation of infective *C. parvum* oocysts. Importantly, the authors of the study were also able to cryogenically preserve *C. parvum*-infected COLO-680 N cells and saw production of oocysts return three days after resuscitation in culture. The authors have also adapted their platform to a bioreactor system, which produced infectious oocysts for up to four months [115].

Primary cells have been studied to support the continuous *in vitro* propagation of *Cryptosporidium* spp. but remain a challenging alternative. These culturing systems recapitulate a more physiologically relevant environment with multiple cell lineages, whereas many of the traditional phenotypic assays only rely upon transformed or immortalized cell lines in a simple monolayer. Initial studies using primary human and bovine intestinal epithelial cells (IECs) demonstrated the short-term ability (<2 days) to support the growth of *C. hominis* and *C. parvum*, respectively [116]. Another study investigating primary human IECs showed a method to maintain longer term viability of the freshly isolated IEC host cells (at least 60 days), but only followed *C. parvum* infection for 5 days [117]. Though primary cells may allow for meaningful study of *Cryptosporidium* infection, they are difficult to obtain and likely do not allow for continuous culture; thus, primary cells may be best suited for small-scale biological studies and not high-throughput drug compound assays.

A further advancement on the concept of primary cells for *Cryptosporidium* culture, organoids derived from *ex vivo* tissues or intestinal epithelial cells have recently emerged as a promising method for culturing *Cryptosporidium* continuously and in a physiologically relevant manner to study both the life cycle progression and critical host-pathogen interactions. These organoids are functional 3D-cultured intestinal epithelial units that contain multiple cell types (enterocytes, goblet, enteroendocrine, Paneth, and stem cells), recapitulate many important aspects of the intestine, and are capable of regeneration and continuous growth [118]. Zhang *et al*. were the first to demonstrate that enteroids isolated from both immunocompetent adult and neonatal mice could be used as an *ex vivo* culture system with purified *C. parvum* sporozoites [119]. Interestingly, although only immunocompromised mice are susceptible to *C. parvum* infection *in vivo* by oral gavage, the enteroids derived from immunocompetent adult mice were successfully infected with *C. parvum* sporozoites, although the authors noted inhibition of enteroid propagation after infection. A subsequent study from Baydoun *et al*. looked at colonic explants from adult SCID mice and were able to successfully co-culture infected explants for 27 days (35 days uninfected) [120]. Yet another study looked at human intestinal and lung organoids derived from adult epithelial stem cells to support *Cryptosporidium* growth [121]. Importantly, these organoids were derived from healthy human tissue, and were able to support continuous culture of the parasites for 28 days, although the number of parasites and oocysts formed decreased over time.

Last year, a further advance was reported by Wilke *et al*. in which they detail a method that combines a stem-cell-derived spheroid culture with a modified air-liquid-interface (ALI) system to create a platform that supports long-term *in vitro* growth and complete development of *C. parvum* [122]. ALI is a method of culturing epithelial cells with the basal surface touching liquid medium and the apical side exposed to air, enabling the cells to differentiate into a pseudostratified cell layer [123]. The authors achieved this through use of a transwell microtiter plate, and found that the ALI monolayers were able to support *C. parvum* growth for at least 20 days, with 10-fold more amplification of parasite growth than infected IECs grown on transwell plates without ALI conditions [124]. ALI culture conditions induced expansion and differentiation from stem cells that resemble cell lineages normally found in the intestine and supported continual renewal of cells. Additionally, RNA-seq revealed gene expression changes in metabolism that may favor *C. parvum* growth, such as preferring oxidative phosphorylation over glycolysis. These key changes may explain the difference in growth of *C. parvum* between ALI and non-ALI IECs grown on transwell plates. Importantly, the authors observed all life stages of *C. parvum* within the ALI culture platform, including production of oocysts that were infective to other cultures and immunocompromised mice. This platform also allowed for *in vitro* generation of transgenic parasite strains, and the first genetic crosses of *Cryptosporidium* performed in culture. The microtiter transwell plate format of the infected ALI cultures is also amenable to scaling for rapid, low-throughput screening or investigating multiple conditions at once.

3.2. Phenotypic assays for pipeline prioritization

Although genetic manipulation of *Cryptosporidium* is now possible, simple, non-resource intensive, and reproducible methods are needed for profiling hits from phenotypic screens. A large proportion of the advanced compounds in Table 1 either have a known or putative target due to their discovery through repositioning from other disease areas; however, there is a large amount of unexplored chemical matter from various HTS efforts for cryptosporidiosis. Ensuring MMOA diversity in the pipeline is important as there are currently no known pathways or targets validated for treating cryptosporidiosis in humans.

Drawing parallels from methods employed by the Medicines for Malaria Venture (MMV), Jumani *et al*. sought to establish a series of phenotypic assays to maintain the cryptosporidiosis pipeline [97,125]. The authors developed the assays using the HCT-8 culturing system and HCI, and roughly followed the parasite life cycle while pulse-treating with the 90% effective concentration (EC_{90}) of each compound as a way to group compounds by their phenotypic effects on growth inhibition. These assays included a host cell invasion assay (1 hr pre-infection +3 hr post-infection compound treatment, then fix, stain, and count), DNA replication assay (compound treatment 3–9 hr post-infection followed by 2 hr of

5-ethynyl-2′-deoxyuridine (EdU) labeling, then fix, stain, and count), parasite egress, and reinvasion assay (addition of compound at 3 hr post-infection, then counting the ratio of parasitophorous vacuoles at 6 hr and 19.5 hr post-infection – timepoints coinciding with the first and second asexual cycles, respectively), and a sexual stage differentiation assay (compound addition at 48 hr post-infection then labeling with a meiosis-specific marker and counting parasites at 72 hr post-infection). The authors profiled 39 compounds in each assay, then used a clustering analysis to identify compound groups with a related MMOA, suggesting that these assays can be used to develop a distinct phenotypic 'fingerprint' that may inform for target identification or MMOA for new compounds. The clusters did not correlate with *in vivo* efficacy in the NSG mouse model, suggesting that multiple MMOAs may produce efficacious compounds – an important aspect of pipeline diversity. The distinct limitations of this study are the small number of compounds, and that the HCT-8 culture platform does not allow for development of the full *C. parvum* life cycle; however, these assays could be applied to other longer-term culture platforms that allow for imaging, and may be a simple way to cluster and prioritize large numbers of compounds from screening campaigns.

Another study sought to look more closely at the life cycle of *C. parvum* in culture to define stage-specific activity of anti-cryptosporidial compounds in the pipeline [126]. The authors built upon their previously established long-term ALI cell culture method [122,127] to profile compounds based on time- and concentration-dependent killing (cidality), as well as their panel of stage-specific monoclonal antibodies [124] in HCT-8 cultures to establish guidelines for profiling compounds based on their stage-specific activity, which may help in determining their mechanism of action. The use of long-term ALI cultures, which allow for completion of the life cycle and oocyst formation, enables a true assessment of 'time-to-kill' rates of compounds because washout and recovery is possible. The authors also sought to clearly delineate the timing and stages of the *C. parvum* life cycle within HCT-8 cells. The benefit of using HCT-8 cultures was two-fold – the cultures maintain a relatively synchronized life cycle and they are more easily manipulated for immunofluorescent imaging. Using a combination of monoclonal antibodies, targeted EdU pulsing, nuclear staining, and monitoring the expression levels of three genes associated with different life stages (asexual stages: trophozoites and type I meronts; sexual stages: type II meronts, microgamonts, and macrogamonts), the authors were able to clearly map the temporal progression of the asexual and sexual life stages in HCT-8 cultures, allowing for precise pulsing of compound treatment and assessment of stage-specific activity. The authors examined a total of five compounds among four classes and found that the putative targets mostly aligned with the observed stage of action. An outlier was benzoxaborole compound AN7973, which showed recovery after treatment with the EC_{90} and 2× EC_{90} and was only effective for a short window of time in pulse-treatment sliding window experiments. This was different from previous reports that showed AN7973 blocked DNA synthesis as determined by EdU incorporation [83], though the previous study used a higher concentration and a longer treatment window. Additionally, AN7973 has a long half-life of killing (~9 hr) yet is efficacious in multiple *in vivo* models of cryptosporidiosis, suggesting that the time- and concentration-dependent kinetic studies by Funkhouser-Jones, *et al*. may be useful to estimate exposure levels for *in vivo* activity, though this has not been proven. More compounds with diverse MMOAs will need to be profiled, but these assays are a much-needed tool for profiling existing and new compounds for prioritization within the cryptosporidiosis pipeline.

4. Conclusion

Since the publication of the GEMS study in 2013 highlighted the unmet medical need to find new cryptosporidiosis therapeutics, many drug discovery programs have commenced and collectively produced >10 potential candidates for clinical development. Of note, these candidates were primarily discovered through phenotypic-based approaches. The ability to screen large chemical libraries against *Cryptosporidium*-infected cells via HTS was developed simultaneously (Figure 2), and the past five years have seen rapid progress toward continuous culturing of the parasites, as well as more realistic and physiologically relevant methods to study the delicate interplay between host and parasite with these new chemical tools.

There are now at least four different HTS assay formats for screening *Cryptosporidium*: HCI, CPE, qRT-PCR, and luciferase-expressing reporter strains (Table 2). All four methods have produced compounds found in the cryptosporidiosis pipeline, and studies that used multiple assay formats showed comparable activities of compounds between formats. These phenotypic-based assays have also been critical as secondary screens to confirm cellular activity of hits from target-based screens.

Advances in culturing methods may have finally reached a tipping point for continuous culture of *Cryptosporidium* spp.

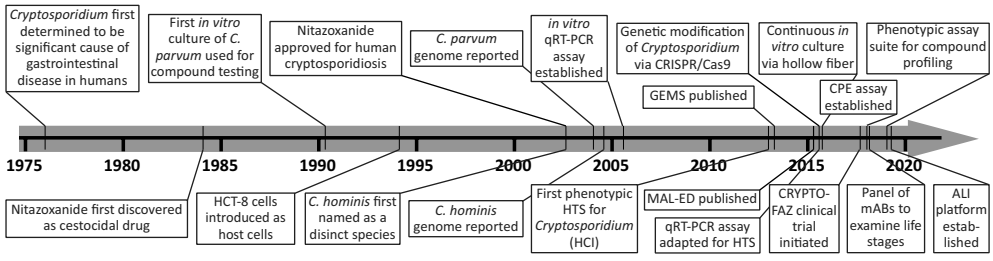

Figure 2. Timeline of milestones for *Cryptosporidium* phenotypic assays.

The longest reported continuous *in vitro C. parvum* culture was a hollow fiber bioreactor with HCT-8 cells for 18 months. This is a tremendous achievement, though acknowledging the drawbacks for reliance on a transformed host cell line, the requirements for specialized equipment and media, and the commitment of personnel and expertise needed to maintain the system. Newer methods using enteroids or differentiated stem cells are more amenable to studies in microtiter plates and offer a promising and sustainable way to profile small-molecule compounds against a continuous culture of *C. parvum*.

The many advances in screening methods to find new compounds with anti-cryptosporidial activity and advances in culturing of *Cryptosporidium* have culminated in the advent of new phenotypic assays to profile and prioritize these compounds. A robust and diverse pipeline of potential therapeutics for cryptosporidiosis has been established, and as compounds inevitably attrit in compound progression, new compounds will need to be assessed and prioritized by MMOA. The suite of assays developed by Jumani *et al*. and Funkhouser-Jones *et al*. provide a reliable framework for categorizing compounds by their phenotypic effects throughout the *C. parvum* life cycle.

5. Expert opinion

The drug discovery field for *Cryptosporidium* is still in its nascent phase compared to many neglected tropical diseases. Despite many challenges unique to *Cryptosporidium* biology, a great number of promising advances have been made in a short period of time. Researchers looking to develop drugs for cryptosporidiosis have been able to draw parallels from other closely related fields (e.g., malaria or kinetoplastid diseases). Phenotypic screening was prioritized due to its success in other fields, as well as a focus on validated targets from other pathogens with homologs in *Cryptosporidium*. Phenotypic screening of focused or repositioned chemical collections as well as larger diversity sets have generated a wealth of new chemical tools with diverse targets and mechanisms of action. It is therefore fitting that the most recent advances in *Cryptosporidium* research have been largely centered around phenotypic assays to profile compounds, as well as methods for continuous *in vitro* culture to potentially allow for target ID and mechanistic studies.

The post-genomic era has seen a return to phenotypic screening for much of the pharmaceutical industry and academia [128]. As *Cryptosporidium* drug discovery has largely existed in the post-genomic era but without many genetic tools, the main strategy has been phenotypic-based screening. To date, clofazimine is the only compound from these efforts to be advanced to clinical trials for cryptosporidiosis, but the molecular target remains unvalidated. As such, there is no clinically validated target for cryptosporidiosis, except for pyruvate:ferredoxin oxidoreductase – the putative target of nitazoxanide. The ability to genetically manipulate *Cryptosporidium* will be a vital tool for identifying or validating targets for compounds in the cryptosporidiosis drug discovery pipeline, previously unexplored screen hits, and new compounds discovered in future screening campaigns. Newly discovered or validated targets may present opportunities for additional target-based screens, creating a back-and-forth dance as more knowledge comes to light about *Cryptosporidium* biology. Importantly, many targets may remain unidentified as previous phenotypic screening approaches have been conducted under conditions that do not recapitulate natural host-pathogen interactions.

The use of HCT-8 or other similar immortalized cell lines as the host for *Cryptosporidium* cultures has been a linchpin for HTS for anti-cryptosporidial compounds. This culturing method is simple, reliable, and adaptable to automation and many readout formats, but fails to support development of all life stages, and therefore the cultures arrest after ~4 days. ALI transwell cultures may be an extremely valuable low- to medium-throughput assay to support profiling of reasonable numbers of compounds, continuous growth, and completion of the *C. parvum* life cycle, and a more physiologically relevant environment in which to perform studies. The completion of the life cycle and development of infectious oocysts may be dependent upon the host cells existing in a polarized or pseudostratified structure, which is achieved through creating a biphasic environment (e.g., transwells with upper and lower chambers, hollow fiber cartridges with the extra-capillary space outside of the fibers, and the silk cocoons with a fibrous basal structure and hollow center). Gene expression and metabolomic data suggest that *Cryptosporidium* induces and is greatly affected by host cell metabolism changes [122,129], which is unsurprising due to the nature of *Cryptosporidium*'s dependence on host-derived biosynthetic pathways for survival. Stem-cell derived IECs grown under ALI conditions may closely mimic the metabolic signatures that *Cryptosporidium* would encounter *in vivo* because they differentiate into cell lineages found in the intestine, and the gene expression profiles of IECs under ALI conditions are much more conducive to *C. parvum* growth than IECs grown under non-ALI conditions.

An added complication to both drug discovery and the study of *Cryptosporidium* biology is the lack of a successful cryopreservation method. Thus, laboratory strains are maintained by continual passage through an animal host. Because sexual replication occurs within a single host, there are likely genetic selections that occur that are specific to the host species. The inability to easily isolate and profile a clonal population of *Cryptosporidium* spp., along with the likely genetic drift over time within reference strains, may introduce variables that convolute the translation of drug mechanisms in a clinical setting [130].

Although this review has not focused on the advancements in *in vivo* models of cryptosporidiosis, the demonstration of compound efficacy in an animal model of disease is a crucial step in the drug development process. The clinical relevance of many of the models commonly used is still unproven, and there are many instances in which those data between models does not agree (e.g., clofazimine is potent in the IFNγ KO mouse model, but shows no effect in the NSG mouse model even at high doses) [67,97]. Though not fully understood, the host immune system plays a large role in controlling *Cryptosporidium* infection, and the difference in immunocompetency between the two models may explain the discrepancy

in activity. It is unknown which model will most closely translate into clinical efficacy in the target populations (e.g., young children, or HIV-positive patients) [131,132]. Animal models with diarrhea (calf and piglet) may be the most relevant with regards to dosing and pharmacokinetics, but these models are generally reserved for later stage compounds and should not be used to prioritize early leads. Disease models with diarrhea are also important for examining the relationship between elimination of clinical symptoms (i.e., diarrhea) and elimination of oocyst shedding. Elimination of diarrhea provides symptom relief and can allow for rehydration treatment of the patient while elimination of parasite shedding may prevent transmission of disease; as such, both endpoints are important for developing a therapeutic for cryptosporidiosis.

Phenotypic screening has been the cornerstone of drug discovery for *Cryptosporidium* and will most likely continue to be an essential element. Genetic tools are just now starting to be more widely adopted within the field, but phenotypic assays for drug screening and profiling will be needed to work in concert to validate targets. Importantly, stem-cell-derived ALI cultures may provide a platform for genetically modified *Cryptosporidium* strains *in vitro*, and because *Cryptosporidium* is able to undergo its entire life cycle within this platform, genetic crosses are possible – a unique advantage over other apicomplexan parasites. Finally, long-term cultures are also an essential development, and may lead to the possibility of obtaining drug-resistant mutants *in vitro* for target identification studies, generation of single cell clones, and the ability to maintain many transgenic lines without the need to passage through animal hosts. Five years ago, the first report of genetic modification of *Cryptosporidium* was a landmark achievement, and because of the continued advances in culturing and phenotypic assays, the next five years are sure to see multiple compounds advance into clinical development.

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References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. UNICEF W, World Bank, UN-DESA population division, levels and trends in child mortality report 2019 estimates developed by the un inter-agency group for child mortality estimation. 2019.
2. Kotloff KL, Nataro JP, Blackwelder WC, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the global enteric multicenter study, GEMS): a prospective, case-control study. Lancet. 2013;382(9888): 209–222.
•• **Important epidemiological study that showed the high burden of cryptosporidiosis in children 0-24 months of age.**
3. Levine MM, Nasrin D, Acacio S, et al. Diarrhoeal disease and subsequent risk of death in infants and children residing in low-income and middle-income countries: analysis of the GEMS case-control study and 12-month GEMS-1A follow-on study. Lancet Glob Health. 2020;8(2):e204–e214.
4. Costa D, Razakandrainibe R, Sautour M, et al. Human cryptosporidiosis in immunodeficient patients in France (2015-2017). Exp Parasitol. 2018;192:108–112.
5. Ghoshal U, Ranjan P, Dey A, et al. Intestinal cryptosporidiosis in renal transplant recipients: prevalence, species detection and comparative evaluation of SSU rRNA and Cryptosporidium oocyst wall protein genes. Indian J Med Microbiol. 2018;36 (2):247–250.
6. Osman M, Benamrouz S, Guyot K, et al. High association of Cryptosporidium spp. infection with colon adenocarcinoma in Lebanese patients. PLoS One. 2017;12(12):e0189422.
7. Feng Y, Ryan UM, Xiao L. Genetic diversity and population structure of cryptosporidium. Trends Parasitol. 2018;34(11):997–1011.
8. Shaw HJ, Innes EA, Morrison LJ, et al. Long-term production effects of clinical cryptosporidiosis in neonatal calves. Int J Parasitol. 2020;50(5):371–376.
9. Leitch GJ, He Q. Cryptosporidiosis-an overview. J Biomed Res. 2012;25(1):1–16.
10. Rossignol JF, Maisonneuve H. Nitazoxanide in the treatment of taenia saginata and hymenolepis nana infections. Am J Trop Med Hyg. 1984;33(3):511–512.
11. White AC Jr. Nitazoxanide: an important advance in anti-parasitic therapy. Am J Trop Med Hyg. 2003;68(4):382–383.
12. Theodos CM, Griffiths JK, D'Onfro J, et al. Efficacy of nitazoxanide against Cryptosporidium parvum in cell culture and in animal models. Antimicrob Agents Chemother. 1998;42(8):1959–1965.
13. Rossignol JF, Ayoub A, Ayers MS. Treatment of diarrhea caused by Cryptosporidium parvum: a prospective randomized, double-blind, placebo-controlled study of nitazoxanide. J Infect Dis. 2001;184 (1):103–106.
14. Amadi B, Mwiya M, Musuku J, et al. Effect of nitazoxanide on morbidity and mortality in Zambian children with cryptosporidiosis: a randomised controlled trial. Lancet. 2002;360 (9343):1375–1380.
15. Rossignol JF, Hidalgo H, Feregrino M, et al. A double-'blind' placebo-controlled study of nitazoxanide in the treatment of cryptosporidial diarrhoea in AIDS patients in Mexico. Trans R Soc Trop Med Hyg. 1998;92(6):663–666.
16. Amadi B, Mwiya M, Sianongo S, et al. High dose prolonged treatment with nitazoxanide is not effective for cryptosporidiosis in HIV positive Zambian children: a randomised controlled trial. BMC Infect Dis. 2009;9(1):195.
17. Tyzzer E. A sporozoan found in the peptic glands of the common mouse. Pro Soc Exp Biol Med. 1907;5(1):12–13.
18. Nime FA, Burek JD, Page DL, et al. Acute enterocolitis in a human being infected with the protozoan Cryptosporidium. Gastroenterology. 1976;70(4):592–598.
19. Platts-Mills JA, Babji S, Bodhidatta L, et al. Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). Lancet Glob Health. 2015;3(9): e564–75.
•• **Second epidemiological study highlighting the large impact of cryptosporidiosis in resource-limited countries.**
20. Striepen B. Parasitic infections: time to tackle cryptosporidiosis. Nature. 2013;503(7475):189–191.

21. Swinney DC, Anthony J. How were new medicines discovered? Nat Rev Drug Discov. 2011;10(7):507–519.
22. Mason LE The role of phenotypic screening in drug discovery. 2017.
23. Abrahamsen MS, Templeton TJ, Enomoto S, et al. Complete genome sequence of the apicomplexan, Cryptosporidium parvum. Science. 2004;304(5669):441–445.
24. Xu P, Widmer G, Wang Y, et al. The genome of Cryptosporidium hominis. Nature. 2004;431(7012):1107–1112.
25. Hedstrom L. Cryptosporidium: a first step toward tractability. Trends Parasitol. 2015;31(9):401–402.
26. Striepen B, Pruijssers AJ, Huang J, et al. Gene transfer in the evolution of parasite nucleotide biosynthesis. Proc Natl Acad Sci U S A. 2004;101(9):3154–3159.
27. Vinayak S, Pawlowic MC, Sateriale A, et al. Genetic modification of the diarrhoeal pathogen Cryptosporidium parvum. Nature. 2015;523(7561): 477–480.

•• **First report of successful genetic modification of Cryptosporidium parvum.**

28. Tandel J, English ED, Sateriale A, et al. Life cycle progression and sexual development of the apicomplexan parasite Cryptosporidium parvum. Nat Microbiol. 2019;4(12):2226–2236.
29. Arnold SLM, Choi R, Hulverson MA, et al. Necessity of bumped kinase inhibitor gastrointestinal exposure in treating cryptosporidium infection. J Infect Dis. 2017;216(1):55–63.
30. Schnyder M, Kohler L, Hemphill A, et al. Prophylactic and therapeutic efficacy of nitazoxanide against Cryptosporidium parvum in experimentally challenged neonatal calves. Vet Parasitol. 2009;160 (1–2):149–154.
31. Janes J, Young ME, Chen E, et al. The ReFRAME library as a comprehensive drug repurposing library and its application to the treatment of cryptosporidiosis. Proc Natl Acad Sci U S A. 2018;115(42):10750–10755.
32. Lee S, Ginese M, Girouard D, et al. Piperazine-derivative mmv665917: an effective drug in the diarrheic piglet model of cryptosporidium hominis. J Infect Dis. 2019;220(2):285–293.
33. Stebbins E, Jumani RS, Klopfer C, et al. Clinical and microbiologic efficacy of the piperazine-based drug lead MMV665917 in the dairy calf cryptosporidiosis model. PLoS Negl Trop Dis. 2018;12(1): e0006183.
34. Sharling L, Liu X, Gollapalli DR, et al. A screening pipeline for antiparasitic agents targeting cryptosporidium inosine monophosphate dehydrogenase. PLoS Negl Trop Dis. 2010;4(8):e794.
35. Johnson CR, Gorla SK, Kavitha M, et al. Phthalazinone inhibitors of inosine-5′-monophosphate dehydrogenase from Cryptosporidium parvum. Bioorg Med Chem Lett. 2013;23(4):1004–1007.
36. Jin Z, Ma J, Zhu G, et al. Discovery of novel anti-cryptosporidial activities from natural products by in vitro high-throughput phenotypic screening. Front Microbiol. 2019;10:1999.
37. Tzipori S, Rand W, Griffiths J, et al. Evaluation of an animal model system for cryptosporidiosis: therapeutic efficacy of paromomycin and hyperimmune bovine colostrum-immunoglobulin. Clin Diagn Lab Immunol. 1994;1(4):450–463.
38. Lee S, Beamer G, Tzipori S. The piglet acute diarrhea model for evaluating efficacy of treatment and control of cryptosporidiosis. Hum Vaccin Immunother. 2019;15(6):1445–1452.
39. Zambriski JA, Nydam DV, Wilcox ZJ, et al. Cryptosporidium parvum: determination of ID_{50} and the dose-response relationship in experimentally challenged dairy calves. Vet Parasitol. 2013;197 (1–2):104–112.
40. Riggs MW, Schaefer DA. Calf clinical model of cryptosporidiosis for efficacy evaluation of therapeutics. Methods Mol Biol. 2020;2052:253–282.
41. Zambriski JA, Nydam DV, Bowman DD, et al. Description of fecal shedding of Cryptosporidium parvum oocysts in experimentally challenged dairy calves. Parasitol Res. 2013;112(3):1247–1254.
42. Chen W, Harp JA, Harmsen AG. Requirements for CD4+ cells and gamma interferon in resolution of established Cryptosporidium parvum infection in mice. Infect Immun. 1993;61(9):3928–3932.
43. Ungar BL, Kao TC, Burris JA, et al. Cryptosporidium infection in an adult mouse model. Independent roles for IFN-gamma and CD4+ T lymphocytes in protective immunity. J Immunol. 1991;147 (3):1014–1022.
44. You X, Mead JR. Characterization of experimental Cryptosporidium parvum infection in IFN-gamma knockout mice. Parasitology. 1998;117(Pt 6):525–531.
45. Urban JF Jr., Fayer R, Chen SJ, et al. IL-12 protects immunocompetent and immunodeficient neonatal mice against infection with Cryptosporidium parvum. J Immunol. 1996;156(1):263–268.
46. Campbell LD, Stewart JN, Mead JR. Susceptibility to Cryptosporidium parvum infections in cytokine- and chemokine-receptor knockout mice. J Parasitol. 2002;88 (5):1014–1016.
47. Jumani RS, Bessoff K, Love MS, et al. A novel piperazine-based drug lead for cryptosporidiosis from the medicines for malaria venture open-access malaria box. Antimicrob Agents Chemother. 2018;62 (4):e01505-17.
48. Mead JR, Arrowood MJ, Sidwell RW, et al. Chronic Cryptosporidium parvum infections in congenitally immunodeficient SCID and nude mice. J Infect Dis. 1991;163(6):1297–1304.
49. McDonald V, Stables R, Warhurst DC, et al. In vitro cultivation of Cryptosporidium parvum and screening for anticryptosporidial drugs. Antimicrob Agents Chemother. 1990;34(8):1498–1500.
50. Upton SJ, Tilley M, Brillhart DB. Comparative development of Cryptosporidium parvum (Apicomplexa) in 11 continuous host cell lines. FEMS Microbiol Lett. 1994;118(3):233–236.
51. Flanigan TP, Aji T, Marshall R, et al. Asexual development of Cryptosporidium parvum within a differentiated human enterocyte cell line. Infect Immun. 1991;59(1):234–239.
52. Gut J, Petersen C, Nelson R, et al. Cryptosporidium parvum: in vitro cultivation in madin-darby canine kidney cells. J Protozool. 1991;38 (6):72s–73s.
53. Buraud M, Forget E, Favennec L, et al. Sexual stage development of cryptosporidia in the Caco-2 cell line. Infect Immun. 1991;59 (12):4610–4613.
54. Villacorta I, de Graaf D, Charlier G, et al. Complete development of Cryptosporidium parvum in MDBK cells. FEMS Microbiol Lett. 1996;142(1):129–132.
55. Karanis P, Aldeyarbi HM. Evolution of Cryptosporidium in vitro culture. Int J Parasitol. 2011;41(12):1231–1242.
56. Varughese EA, Bennett-Stamper CL, Wymer LJ, et al. A new in vitro model using small intestinal epithelial cells to enhance infection of Cryptosporidium parvum. J Microbiol Methods. 2014;106:47–54.
57. Zhu G, Marchewka MJ, Woods KM, et al. Molecular analysis of a Type I fatty acid synthase in Cryptosporidium parvum. Mol Biochem Parasitol. 2000;105(2):253–260.
58. Arrowood MJ, Mead JR, Xie L, et al. In vitro anticryptosporidial activity of dinitroaniline herbicides. FEMS Microbiol Lett. 1996;136 (3):245–249.
59. Arrowood MJ, Xie LT, Hurd MR. In vitro assays of maduramicin activity against Cryptosporidium parvum. J Eukaryot Microbiol. 1994;41(5):23s.
60. You X, Arrowood MJ, Lejkowski M, et al. In vitro evaluation of anticryptosporidial agents using MDCK cell culture and chemiluminescence immunoassay. J Eukaryot Microbiol. 1996;43(5):87s.
61. Armson A, Meloni BP, Reynoldson JA, et al. Assessment of drugs against Cryptosporidium parvum using a simple in vitro screening method. FEMS Microbiol Lett. 1999;178(2):227–233.
62. Bessoff K, Sateriale A, Lee KK, et al. Drug repurposing screen reveals FDA-approved inhibitors of human HMG-CoA reductase and isoprenoid synthesis that block Cryptosporidium parvum growth. Antimicrob Agents Chemother. 2013;57(4): 1804–1814.

•• **First report of a phenotypic high-throughput screen for Cryptosporidium parvum and establishment of a high-content imaging assay.**

63. Llovo J, Lopez A, Fabregas J, et al. Interaction of lectins with Cryptosporidium parvum. J Infect Dis. 1993;167(6):1477–1480.
64. Gut J, Nelson RG. Cryptosporidium parvum: synchronized excystation in vitro and evaluation of sporozoite infectivity with a new lectin-based assay. J Eukaryot Microbiol. 1999;46(5):56s–57s.

65. Bessoff K, Spangenberg T, Foderaro JE, et al. Identification of Cryptosporidium parvum active chemical series by Repurposing the open access malaria box. Antimicrob Agents Chemother. 2014;58(5):2731–2739.
66. Love MS, McNamara CW. High-content screening for cryptosporidium drug discovery. Methods Mol Biol. 2020;2052:303–317.
67. Love MS, Beasley FC, Jumani RS, et al. A high-throughput phenotypic screen identifies clofazimine as a potential treatment for cryptosporidiosis. PLoS Negl Trop Dis. 2017;11(2):e0005373.
68. Nachipo P, Hermann D, Quinnan G, et al. Evaluating the safety, tolerability, pharmacokinetics and efficacy of clofazimine in cryptosporidiosis (CRYPTOFAZ): study protocol for a randomized controlled trial. Trials. 2018;19(1):456.
69. Iroh Tam PY, Arnold SLM, Barrett LK, et al. Clofazimine for treatment of cryptosporidiosis in HIV-infected adults (CRYPTOFAZ): an experimental medicine, randomized, double-blind, placebo-controlled phase 2a trial. Clin Infect Dis. 2020. DOI:10.1093/cid/ciaa421.
70. Chen JJ, Rateb ME, Love MS, et al. Herbicidins from streptomyces sp. CB01388 showing anti- cryptosporidium activity. J Nat Prod. 2018;81(4):791–797.
71. Cai X, Woods KM, Upton SJ, et al. Application of quantitative real-time reverse transcription-PCR in assessing drug efficacy against the intracellular pathogen Cryptosporidium parvum in vitro. Antimicrob Agents Chemother. 2005;49(11):4437–4442.
72. Zhang H, Zhu G. Quantitative RT-PCR assay for high-throughput screening (HTS) of drugs against the growth of Cryptosporidium parvum in vitro. Front Microbiol. 2015;6:991.
- **Establishment of high-throughput screening of compounds against C. parvum via qRT-PCR.**
73. Zhang H, Zhu G. High-throughput screening of drugs against the growth of cryptosporidium parvum in vitro by qRT-PCR. Methods Mol Biol. 2020;2052:319–334.
74. Guo F, Zhang H, McNair NN, et al. The existing drug vorinostat as a new lead against cryptosporidiosis by targeting the parasite histone deacetylases. J Infect Dis. 2018;217(7):1110–1117.
75. Eltahan R, Guo F, Zhang H, et al. Discovery of ebselen as an inhibitor of Cryptosporidium parvum glucose-6-phosphate isomerase (CpGPI) by high-throughput screening of existing drugs. Int J Parasitol Drugs Drug Resist. 2018;8(1):43–49.
76. Eltahan R, Guo F, Zhang H, et al. The action of the hexokinase inhibitor 2-deoxy-d-glucose on cryptosporidium parvum and the discovery of activities against the parasite hexokinase from marketed drugs. J Eukaryot Microbiol. 2019;66(3):460–468.
77. Chao AT, Lee BH, Wan KF, et al. Development of a cytopathic effect-based phenotypic screening assay against Cryptosporidium. ACS Infect Dis. 2018;4(4): 635–645.
- **Establishment of HTS of compounds against Cryptosporidium spp. via a cytopathic effect assay.**
78. Manjunatha UH, Vinayak S, Zambriski JA, et al. A Cryptosporidium PI(4)K inhibitor is a drug candidate for cryptosporidiosis. Nature. 2017;546(7658):376–380.
79. Hulverson MA, Vinayak S, Choi R, et al. Bumped-kinase inhibitors for cryptosporidiosis therapy. J Infect Dis. 2017;215(8):1275–1284.
80. Hulverson MA, Choi R, Arnold SLM, et al. Advances in bumped kinase inhibitors for human and animal therapy for cryptosporidiosis. Int J Parasitol. 2017;47(12):753–763.
81. Lim SP, Wang QY, Noble CG, et al. Ten years of dengue drug discovery: progress and prospects. Antiviral Res. 2013;100 (2):500–519.
82. McNamara CW, Lee MC, Lim CS, et al. Targeting Plasmodium PI(4)K to eliminate malaria. Nature. 2013;504(7479):248–253.
83. Lunde CS, Stebbins EE, Jumani RS, et al. Identification of a potent benzoxaborole drug candidate for treating cryptosporidiosis. Nat Commun. 2019;10(1):2816.
84. Huang J, Mullapudi N, Lancto CA, et al. Phylogenomic evidence supports past endosymbiosis, intracellular and horizontal gene transfer in Cryptosporidium parvum. Genome Biol. 2004;5(11):R88.
85. Sateriale A, Striepen B. Beg, borrow and steal: three aspects of horizontal gene transfer in the protozoan parasite, Cryptosporidium parvum. PLoS Pathog. 2016;12(3):e1005429.
86. Murphy RC, Ojo KK, Larson ET, et al. Discovery of potent and selective inhibitors of calcium-dependent protein kinase 1 (CDPK1) from C. parvum and T. gondii. ACS Med Chem Lett. 2010;1(7):331–335.
87. Chen XM, O'Hara SP, Huang BQ, et al. Apical organelle discharge by Cryptosporidium parvum is temperature, cytoskeleton, and intracellular calcium dependent and required for host cell invasion. Infect Immun. 2004;72(12):6806–6816.
88. Ojo KK, Larson ET, Keyloun KR, et al. Toxoplasma gondii calcium-dependent protein kinase 1 is a target for selective kinase inhibitors. Nat Struct Mol Biol. 2010;17(5):602–607.
89. Larson ET, Ojo KK, Murphy RC, et al. Multiple determinants for selective inhibition of apicomplexan calcium-dependent protein kinase CDPK1. J Med Chem. 2012;55(6):2803–2810.
90. Choi R, Hulverson MA, Huang W, et al. Bumped Kinase Inhibitors as therapy for apicomplexan parasitic diseases: lessons learned. Int J Parasitol. 2020;50(5):413–422.
91. Castellanos-Gonzalez A, Sparks H, Nava S, et al. A novel calcium-dependent kinase inhibitor, bumped kinase inhibitor 1517, cures cryptosporidiosis in immunosuppressed mice. J Infect Dis. 2016;214(12):1850–1855.
92. Lendner M, Böttcher D, Delling C, et al. A novel CDPK1 inhibitor–a potential treatment for cryptosporidiosis in calves? Parasitol Res. 2015;114(1):335–336.
93. Castellanos-Gonzalez A, White AC Jr., Ojo KK, et al. A novel calcium-dependent protein kinase inhibitor as a lead compound for treating cryptosporidiosis. J Infect Dis. 2013;208(8):1342–1348.
94. Kato N, Comer E, Sakata-Kato T, et al. Diversity-oriented synthesis yields novel multistage antimalarial inhibitors. Nature. 2016;538 (7625):344–349.
95. Baragaña B, Forte B, Choi R, et al. Lysyl-tRNA synthetase as a drug target in malaria and cryptosporidiosis. Proc Natl Acad Sci U S A. 2019;116(14):7015–7020.
96. Buckner FS, Ranade RM, Gillespie JR, et al. Optimization of methionyl tRNA-synthetase inhibitors for treatment of Cryptosporidium infection. Antimicrob Agents Chemother. 2019;63(4). DOI:10.1128/AAC.02061-18.
97. Jumani RS, Hasan MM, Stebbins EE, et al. A suite of phenotypic assays to ensure pipeline diversity when prioritizing drug-like Cryptosporidium growth inhibitors. Nat Commun. 2019;10(1): 1862.
- **Established a series of in vitro assays to profile anti-cryptosporidial compounds by mode of action.**
98. Umejiego NN, Li C, Riera T, et al. Cryptosporidium parvum IMP dehydrogenase: identification of functional, structural, and dynamic properties that can be exploited for drug design. J Biol Chem. 2004;279(39):40320–40327.
99. Umejiego NN, Gollapalli D, Sharling L, et al. Targeting a prokaryotic protein in a eukaryotic pathogen: identification of lead compounds against cryptosporidiosis. Chem Biol. 2008;15(1):70–77.
100. Gorla SK, McNair NN, Yang G, et al. Validation of IMP dehydrogenase inhibitors in a mouse model of cryptosporidiosis. Antimicrob Agents Chemother. 2014;58(3):1603–1614.
101. Gorla SK, Kavitha M, Zhang M, et al. Optimization of benzoxazole-based inhibitors of Cryptosporidium parvum inosine 5'-monophosphate dehydrogenase. J Med Chem. 2013;56 (10):4028–4043.
102. Kim Y, Makowska-Grzyska M, Gorla SK, et al. Structure of Cryptosporidium IMP dehydrogenase bound to an inhibitor with in vivo antiparasitic activity. Acta Crystallogr F Struct Biol Commun. 2015;71(Pt 5):531–538.
103. Fritzler JM, Zhu G. Novel anti-Cryptosporidium activity of known drugs identified by high-throughput screening against parasite fatty acyl-CoA binding protein (ACBP). J Antimicrob Chemother. 2012;67(3):609–617.
104. Guo F, Zhang H, Fritzler JM, et al. Amelioration of Cryptosporidium parvum infection in vitro and in vivo by targeting parasite fatty acyl-coenzyme A synthetases. J Infect Dis. 2014;209(8):1279–1287.
105. Chattopadhyay S, Mahapatra RK. Identification of adaptive inhibitors of Cryptosporidium parvum fatty acyl-coenzyme a synthetase isoforms by virtual screening. Parasitol Res. 2019;118 (11):3159–3171.

106. Hijjawi NS, Meloni BP, Ng'anzo M, et al. Complete development of Cryptosporidium parvum in host cell-free culture. Int J Parasitol. 2004;34(7):769–777.
107. Hijjawi N, Estcourt A, Yang R, et al. Complete development and multiplication of Cryptosporidium hominis in cell-free culture. Vet Parasitol. 2010;169(1–2):29–36.
108. Arrowood MJ. In vitro cultivation of cryptosporidium species. Clin Microbiol Rev. 2002;15(3):390–400.
109. Morada M, Lee S, Gunther-Cummins L, et al. Continuous culture of Cryptosporidium parvum using hollow fiber technology. Int J Parasitol. 2016;46(1): 21–29.
• **Discovery of in vitro continuous propagation of C. parvum via hollow fiber.**
110. Yarlett N, Morada M. Long-term in vitro culture of Cryptosporidium parvum. Bio-protocol. 2018;8(15):e2947.
111. Yarlett N, Morada M, Gobin M, et al. In vitro culture of Cryptosporidium parvum using hollow fiber bioreactor: applications for simultaneous pharmacokinetic and pharmacodynamic evaluation of test compounds. Methods Mol Biol. 2020;2052:335–350.
112. DeCicco RePass MA, Chen Y, Lin Y, et al. Novel bioengineered three-dimensional human intestinal model for long-term infection of Cryptosporidium parvum. Infect Immun. 2017;85(3). DOI:10.1128/IAI.00731-16.
113. Zhou W, Chen Y, Roh T, et al. Multifunctional bioreactor system for human intestine tissues. ACS Biomater Sci Eng. 2018;4(1):231–239.
114. Miller CN, Josse L, Brown I, et al. A cell culture platform for Cryptosporidium that enables long-term cultivation and new tools for the systematic investigation of its biology. Int J Parasitol. 2018;48(3–4):197–201.
115. Jossé L, Bones AJ, Purton T, et al. A cell culture platform for the cultivation of Cryptosporidium parvum. Curr Protoc Microbiol. 2019;53(1):e80.
116. Hashim A, Mulcahy G, Bourke B, et al. Interaction of Cryptosporidium hominis and Cryptosporidium parvum with primary human and bovine intestinal cells. Infect Immun. 2006;74 (1):99–107.
117. Castellanos-Gonzalez A, Cabada MM, Nichols J, et al. Human primary intestinal epithelial cells as an improved in vitro model for Cryptosporidium parvum infection. Infect Immun. 2013;81 (6):1996–2001.
118. Zachos NC, Kovbasnjuk O, Foulke-Abel J, et al. Human enteroids/colonoids and intestinal organoids functionally recapitulate normal intestinal physiology and pathophysiology. J Biol Chem. 2016;291 (8):3759–3766.
119. Zhang XT, Gong AY, Wang Y, et al. Cryptosporidium parvum infection attenuates the ex vivo propagation of murine intestinal enteroids. Physiol Rep. 2016;4(24):e13060.
120. Baydoun M, Vanneste SB, Creusy C, et al. Three-dimensional (3D) culture of adult murine colon as an in vitro model of cryptosporidiosis: proof of concept. Sci Rep. 2017;7(1):17288.
121. Heo I, Dutta D, Schaefer DA, et al. Modelling Cryptosporidium infection in human small intestinal and lung organoids. Nat Microbiol. 2018;3(7):814–823.
122. Wilke G, Funkhouser-Jones LJ, Wang Y, et al. A stem-cell-derived platform enables complete Cryptosporidium development in vitro and genetic tractability. Cell Host Microbe. 2019;26(1):123–134.e8.
123. Gangatirkar P, Paquet-Fifield S, Li A, et al. Establishment of 3D organotypic cultures using human neonatal epidermal cells. Nat Protoc. 2007;2(1):178–186.
124. Wilke G, Ravindran S, Funkhouser-Jones L, et al. Monoclonal antibodies to intracellular stages of Cryptosporidium parvum define life cycle progression in vitro. mSphere. 2018; 3(3). DOI: 10.1128/mSphere.00124-18.
• **Established a collection of antibodies that are specific to the life stages of C. parvum, an enabling tool for stage-specific profiling of anti-cryptosporidial compounds.**
125. Burrows JN, Duparc S, Gutteridge WE, et al. New developments in anti-malarial target candidate and product profiles. Malar J. 2017;16(1):26.
126. Funkhouser-Jones LJ, Ravindran S, Sibley LD. Defining stage-specific activity of potent new inhibitors of Cryptosporidium parvum growth in vitro. mBio. 2020;11(2):e00052-20.
•• **Established a cell culture platform that allows for in vitro production of C. parvum oocysts and applied the previously established antibody collection to examine stage-specific phenotypes of several anti-cryptosporidial compounds.**
127. Wilke G, Wang Y, Ravindran S, et al. In vitro culture of Cryptosporidium parvum using stem cell-derived intestinal epithelial monolayers. Methods Mol Biol. 2020;2052:351–372.
128. Haasen D, Schopfer U, Antczak C, et al. How phenotypic screening influenced drug discovery: lessons from five years of practice. Assay Drug Dev Technol. 2017;15(6):239–246.
129. Miller CN, Panagos CG, Mosedale WRT, et al. NMR metabolomics reveals effects of Cryptosporidium infections on host cell metabolome. Gut Pathog. 2019;11(1):13.
130. Isaza JP, Galván AL, Polanco V, et al. Revisiting the reference genomes of human pathogenic Cryptosporidium species: reannotation of C. parvum Iowa and a new C. hominis reference. Sci Rep. 2015;5(1):16324.
131. Huston CD, Spangenberg T, Burrows J, et al. A proposed target product profile and developmental cascade for new cryptosporidiosis treatments. PLoS Negl Trop Dis. 2015;9(10):e0003987.
132. Manjunatha UH, Chao AT, Leong FJ, et al. Cryptosporidiosis drug discovery: opportunities and challenges. ACS Infect Dis. 2016;2 (8):530–537.