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Current status and challenges in drug discovery against the globally important zoonotic cryptosporidiosis

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Abstract

The zoonotic cryptosporidiosis is globally distributed, one of the major diarrheal diseases in humans and animals. *Cryptosporidium* oocysts are also one of the major environmental concerns, making it a pathogen that fits well into the One Health concept. Despite its importance, fully effective drugs are not yet available. Anti-cryptosporidial drug discovery has historically faced many unusual challenges attributed to unique parasite biology and technical burdens. While significant progresses have been made recently, anti-cryptosporidial drug discovery still faces a major obstacle: identification of systemic drugs that can be absorbed by patients experiencing watery diarrhea and effectively pass through electron-dense (ED) band at the parasite-host cell interface to act on the epicellular parasite. There may be a need to develop an *in vitro* assay to effectively screen hits/leads for their capability to cross ED band. In the meantime, non-systemic drugs with strong mucoadhesive properties for extended gastrointestinal exposure may represent another direction in developing anti-cryptosporidial therapeutics. For developing both systemic and non-systemic drugs, a non-ruminant animal model exhibiting diarrheal symptoms suitable for routine evaluation of drug absorption and anti-cryptosporidial efficacy may be very helpful.

Keywords: *Cryptosporidium*, Cryptosporidiosis, Drug discovery, Watery diarrhea, Electron-dense band, Drug delivery

The zoonotic *Cryptosporidium* as an important One Health pathogen

Significance of cryptosporidiosis in human and animal health

The enteric *Cryptosporidium* is a globally distributed, water-borne and food-borne diarrheal-causing parasite (Tzipori and Widmer 2000; Checkley et al. 2015). The major symptom of cryptosporidiosis is watery diarrhea that may range from mild to severe or deadly in humans and animals. Currently, there are ~40 recognized *Cryptosporidium* species or genotypes (Feng et al. 2018). Humans are mainly infected by the zoonotic *C. parvum* and anthropogenic *C. hominis*. Patients with weakened or compromised immunity may be infected by other species (e.g., *C. canis, C. felis, C. meleagridis* and *C. xiaoi*) (O'Connor et al. 2011; Adamu et al. 2014; Ryan et al. 2014; Pumipuntu and Piratae 2018). It is also one of the top diarrheal pathogens afflicting children in developing countries with significant negative impact on mortality and growth (Kotloff et al. 2013), resulting in an estimated 4.2 million disability adjusted life years (DALY) lost in children under 5 years old (Khalil et al. 2018). One particular concern is the long-term effects in children, involving growth stunting and cognitive deficits even after the recovery of cryptosporidiosis (Kotloff et al. 2013; Khalil et al. 2018).

Cryptosporidiosis is also a significant health problem in wild and domesticated animals. In farm animals, *Cryptosporidium* is responsible for the severe to deadly

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neonatal diarrhea syndrome of calves and other young ruminants, resulting in considerable direct and indirect economic losses (de Graaf et al. 1999; Olson et al. 2004). Cryptosporidiosis also produces long-term negative effects in animals, including lowering weight gains and production performance in cattle and sheep (Jacobson et al. 2016; Innes et al. 2020; Shaw et al. 2020). For example, beef calves might lose an average of 34 kg at 6 months of age if they had experienced cryptosporidiosis as neonates in comparison to those with no clinical signs of infection (Shaw et al. 2020).

Cryptosporidium as a One Health model pathogen

Oocysts are environmental stage of the parasite responsible for transmission between humans and/or animals. Oocysts contain a wall structure that is highly resistant to chemicals including disinfectants, while the 4 sporozoites within an oocyst wall are reasonably resistant to temperature fluctuation and desiccation, probably related to the presence of stress protectant trehalose (Yu et al. 2010). For these reasons, cryptosporidium oocysts are a significant environmental contaminant, responsible for numerous water-borne outbreaks of cryptosporidiosis around the world (Corso et al. 2003; Chyzheuskaya et al. 2017; Ridderstedt et al. 2018). Long-surviving oocysts may be carried to different places around the world in water and soil to interact with humans and animals, and adapted to new environments and hosts, which complicate the evolution of epidemiology and population structures of cryptosporidiosis (Feng et al. 2018). Therefore, *Cryptosporidium* may serve as one of the model One Health pathogens that impact human, animal and environmental health at regional and global levels (Fig. 1) (Ryan et al. 2016; Innes et al. 2020).

Unique intracellular lifestyle of *Cryptosporidium*

Cryptosporidium is transmitted *via* fecal-oral route, and has a unique epi-cytoplasmic lifestyle (i.e., intracellular, but extra-cytoplasmic) (Prystajecky et al. 2014). When humans or animals ingest oocysts, sporozoites are released from oocysts to invade intestinal epithelial cells, starting its intracellular development. In humans and other mammals, ileum is the major infection site for intestinal cryptosporidium species (Leitch and He 2012; Abdou et al. 2013).

Parasite mainly infects intestinal epithelial cells, where it is separated from the host cell cytosol by an electron-dense (ED) band and associated structures, but embraced by a host cell-derived parasitophorous vacuole membrane (PVM) (Fig. 2) (Elliott and Clark 2000). This epi-cytoplasmic location makes *Cryptosporidium* different from other apicomplexans that reside within the host cell cytosol (e.g., *Eimeria*, *Toxoplasma* and *Plasmodium*).

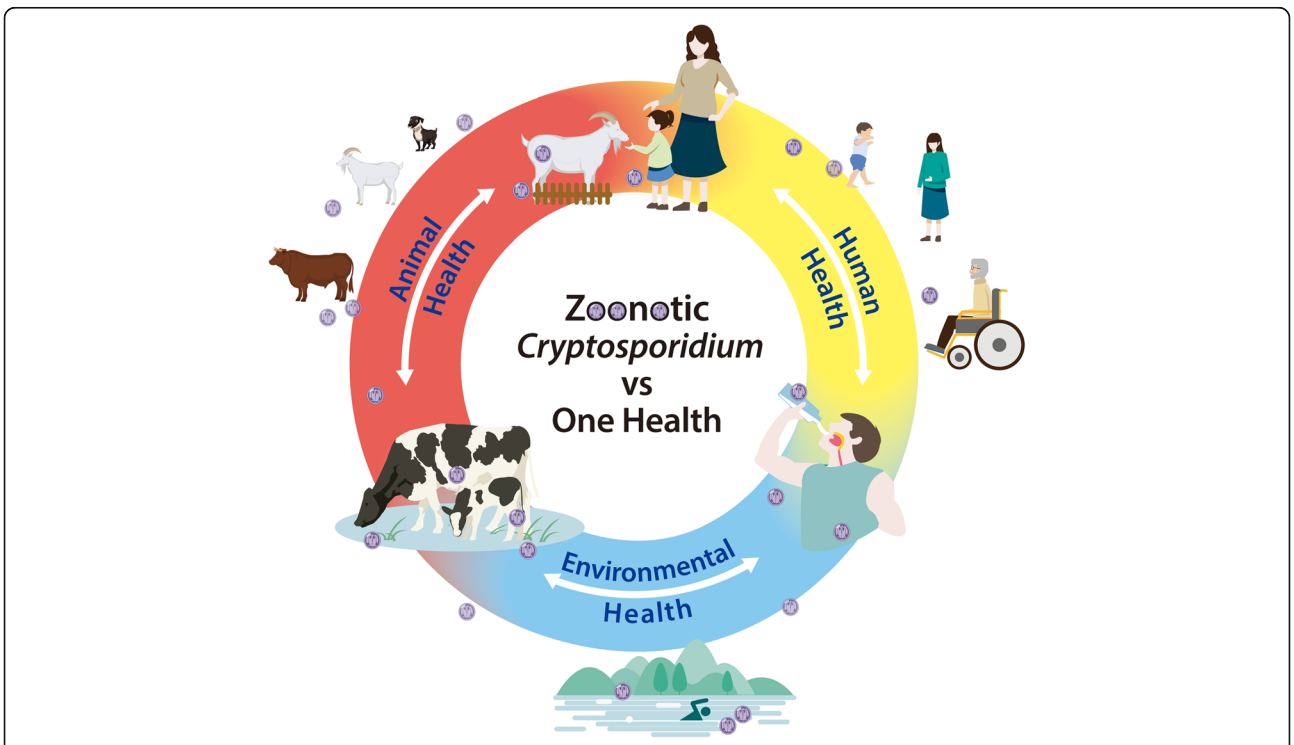

Fig. 1 Interactions and impact of *Cryptosporidium* on human, animal and environmental health under the One Health concept. The stress-resistant oocysts play a central role in transmission of cryptosporidial infections between and among humans and animals *via* direct contact and/or contamination of environment. Credit to Lin Xia and Wenjuan Wang for illustration

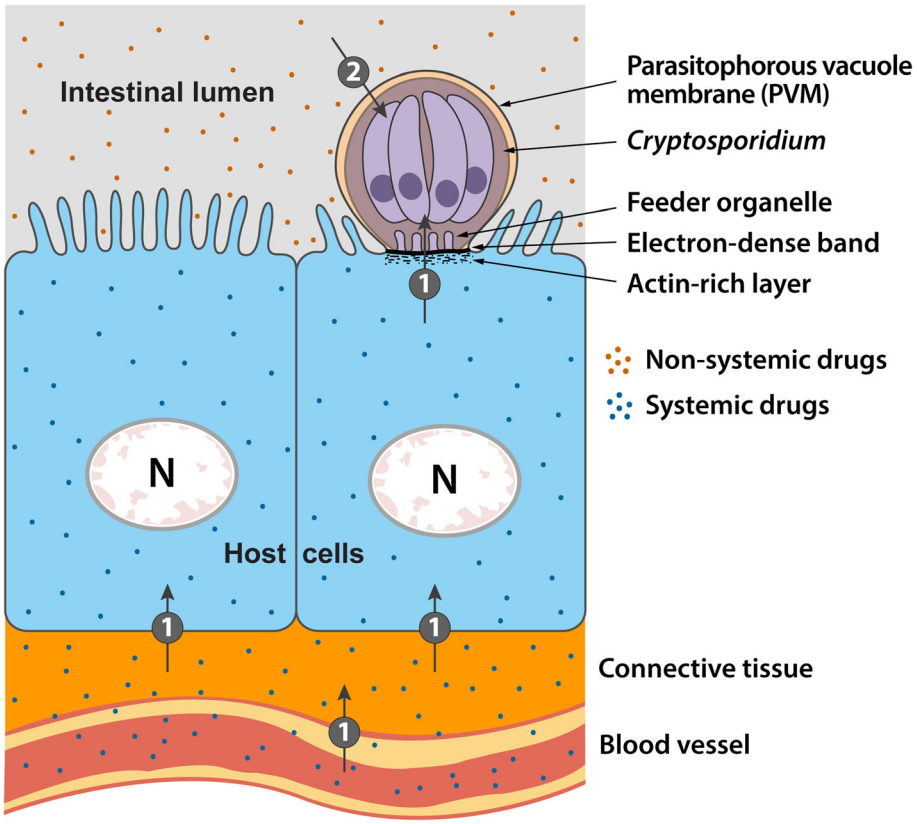

Fig. 2 Illustration of drug delivery routes for systemic and non-systemic drugs to an intracellular *Cryptosporidium* meront that is separated from host epithelial cell cytosol by an electron-dense (ED) band, but embraced by a host cell-derived parasitophorous vacuole membrane (PVM) facing the intestinal lumen. Systemic drugs need to cross through the selective ED band and feeder organelle (route 1), while luminal non-systemic drugs may directly act on parasite through PVM (route 2). A regular 2D culture system is unable to evaluate systemic drug route since the basal surface of host cells is attached to a nonporous plastic surface. Credit to Lin Xia and Wenjuan Wang for illustration

Current status of anti-cryptosporidial drug discovery

Limited treatment options

Despite its medical and veterinary importance, current options to treat cryptosporidiosis in humans and animals are limited. Only nitazoxanide is approved by the Food and Drug Administration (FDA) in the United States to treat cryptosporidiosis in immune-competent individuals, but not for immuno-compromised individuals, such as AIDS patients (Chappell and Okhuysen 2002; White Jr. 2003; Jenkins 2004; Smith and Corcoran 2004; Fox and Saravolatz 2005). However, nitazoxanide is not fully effective (Fox and Saravolatz 2005), and has an ill-defined mechanism of action. There is also no FDA-approved drugs to treat cryptosporidiosis in animals. In some countries, halofuginone lactate (Halocur) is approved for veterinary use in calves and lambs, which displays some anti-cryptosporidial efficacy, but again it's not fully effective in eliminating oocyst production (Klein 2008; Trotz-Williams et al. 2011; Petermann et al. 2014).

Recently discovered anti-cryptosporidial leads and targets

An increasing effort over the past two decades has led to the discovery of several lead compounds with defined targets and anti-cryptosporidial efficacies at lower nanomolar level *in vitro* and low mg/kg/d doses in mouse models. A few of them have also been evaluated in calves. These leads include "compound 5" on lysyl-tRNA synthetase (KRS) (Baragana et al. 2019), BRD7929 on phenylalanyl-tRNA synthetase (PheRS) (Vinayak et al. 2020), inhibitors of bumped-kinase (Arnold et al. 2017; Lee et al. 2018; Huang et al. 2019), "compound 1294" on calcium-dependent protein kinase (CDPK) (Castellanos-Gonzalez et al. 2013), P131 on inosine-5′-mono-phosphate dehydrogenase (IMPDH), (Gorla et al. 2014), KDU731 on phosphatidylinositol-4-OH kinase (PI (4) K) (Manjunatha et al. 2017), triacsin C on acyl-CoA synthetase (ACS) (Guo et al. 2014), vorinostat on histone deacetylase (HDAC) (Guo et al. 2018), and gossypol on lactate dehydrogenase (LDH) (Zhang et al. 2015; Li et al. 2019). However, they are mostly in various preclinical and lead optimization stages. Even if one or more of

these leads (or analogs) are developed into therapeutics in the future, more drug options are still needed to diversify drug targets and for overcoming potential drug resistance.

More recently, a randomized, double-blind, placebo-controlled study on the efficacy of clofazimine for treating cryptosporidiosis in HIV-infected patients was reported. However, the "findings do not support the efficacy of clofazimine for the treatment of cryptosporidiosis in a severely immunocompromised HIV population" (Iroh Tam et al. 2020). This further exemplifies the difficulties in developing anti-cryptosporidial drugs.

Historical challenges and progresses in anti-cryptosporidial drug discovery

Anti-cryptosporidial drug discovery faces additional challenges compared to other apicomplexans. Limiting factors are present at virtually all stages of drug development pipeline from basic research to preclinical investigations (Fig. 3). This section will briefly discuss the historical challenges and progress related to anti-cryptosporidial drug discovery and development.

Limited availability of parasite materials

A major limiting factor impeding research and drug discovery in this particular field is the difficulties in obtaining large number of pure parasite materials for experiments. The zoonotic species *C. parvum* is the most commonly used species in research, for which oocysts can be obtained at a reasonable scale from bovine neonatal calves (up to billions of oocysts), or in limited amounts in some immunodeficient mice (up to millions of oocysts). Oocysts can be isolated from calf feces by gradient centrifugation procedures and further cleaned using chlorine to achieve high purity. Free sporozoites can be obtained by *in vitro* excystation (Arrowood and Sterling 1987; Arrowood and Donaldson 1996; Arrowood 2020). Therefore, oocysts and sporozoites are the only 2 stages for which highly pure materials can be obtained for experimental use. For *C. hominis*, only a gnotobiotic piglet model is available for maintaining and propagating certain parasite isolates in specialized facilities (Lee et al. 2018, 2019).

Lack of continuous *in vitro* cultivation platform for routine use

In vitro culture protocols of *C. parvum* have been established for decades. Parasite oocysts can undergo excystation to release sporozoites that invade host cells and develop for 2–3 days under *in vitro* condition. The *in vitro* growth is mostly limited to asexual stages (i.e., merogony), and up to the formation of macro- and micro-gametes that are unable to fertilize to form zygotes and viable oocysts (Tandel et al. 2019). *In vitro* culture can provide less synchronized meronts and merozoites in host cells for microscopic and molecular

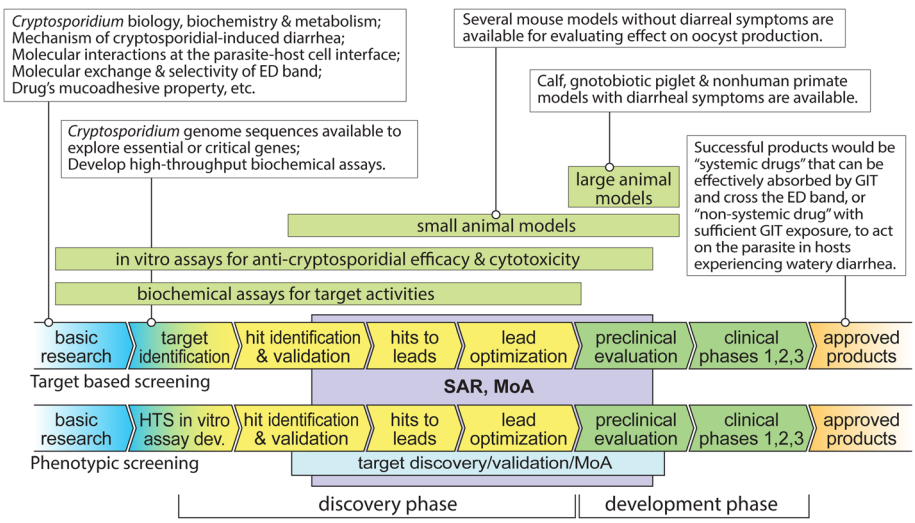

Fig. 3 Illustration and brief annotation of drug discovery pipeline, major assays and models in target-based and phenotypic screening approaches. Yellow and green boxes indicate discovery and developmental phases. In both approaches, structure-activity relationship (SAR) and mode of action (MoA) analysis may be started shortly after hits are identified and extended to later stages. In the phenotypic screening approach, discovery of target by the identified hit may be needed for determining MoA. Biochemical assays and in vitro assays may be developed in the basic research phase and used throughout the discovery phase, and extended into the preclinical phase. Small animal models are used in discovery and preclinical phases, while large animal models are used in preclinical phase. For drug discovery against cryptosporidiosis, it's critical to discover efficacious systemic drugs that can be effectively absorbed by human or animal patients and cross electron-dense (ED) band at the parasite-host cell interface, or non-systemic drugs that have sufficient gastrointestinal tract (GIT) exposure for acting on the epicellular parasite (as illustrated in Fig. 2)

experiments, but is unable to propagate parasite for providing unlimited materials. The inability to continuously grow parasite *in vitro* has been a significant obstacle for both cryptosporidium and coccidia (such as *Eimeria* species) (Muller and Hemphill 2013). For comparison, *Toxoplasma* and *Plasmodium* parasites have a merozoite stage that can be continuously cultured in fibroblasts or erythrocytes *in vitro* (Muller and Hemphill 2013; Szabo and Finney 2017; Bermudez et al. 2018; Duffy and Avery 2018).

More recently, complete life cycle development of *C. parvum* was obtained using a stem-cell-derived platform, which represents a progress milestone in cryptosporidium research (Wilke et al. 2019). Another notable development is the continuous 3D cultivation of *Cryptosporidium* in hollow fiber bioreactors (Morada et al. 2016; Yarlett et al. 2020), and a few bioengineered intestinal models based on silk-protein scaffold, colon explants or lung/small intestine organoids cultured with parasites over weeks (Baydoun et al. 2017; DeCicco RePass et al. 2017; Heo et al. 2018). A more comprehensive review on these organoids and bioengineered intestinal models was reported by Gunasekera et al. (2020). Nonetheless, these models and platforms are not yet optimized for routine production of oocysts, and remain technically challenging for regular laboratory use. However, they raise hope for further optimization and adaptation.

Absence of conventional drug targets

At a basic biology level, cryptosporidium lacks many drug targets present in other apicomplexans because of a highly streamlined metabolism and absence of *de novo* nutrient synthetic pathways (e.g., amino acids, nucleotides and fatty acids), or targets that are highly divergent (e.g., DHFR) (Abrahamsen et al. 2004; Xu et al. 2004; Zhu 2007; Rider Jr. and Zhu 2010). The difficult gene-hunting game was concluded in 2004 after the whole genome sequences of several *Cryptosporidium* species (Abrahamsen et al. 2004; Xu et al. 2004; Rider Jr. and Zhu 2010; Ifeonu et al. 2016), providing opportunities for investigators to intellectually explore various essential or critical proteins and pathways as potential drug targets (see Current status of anti-cryptosporidial drug discovery section above for examples on recently discovered leads and targets). Although not every explored target will lead to a successful clinically validated drug, the more we explore, the higher probability that one or more drugs operating *via* different action modes could be eventually developed.

Available now but still tricky genetic manipulation

At the genetic level, there used to be a lack of tools for ultimate drug target validation. This obstacle is partially resolved by the successful development of CRIPR/Cas9-based genome-editing tools in *C. parvum* and *C. tyzzeri* (Vinayak et al. 2015; Sateriale et al. 2019, 2020). It is now possible to conduct gene knockout to evaluate the essentialness or importance of a gene in cryptosporidium, albeit the tool may not be easily adapted for use by routine laboratories. Conditional gene knockout strategies are also under development for studying essential genes and validating drug targets (Choudhary et al. 2020). The mouse-specific species *C. tyzzeri* is evolutionarily more closely related to *C. parvum* and *C. hominis* than other species that infecting mammals (Sateriale et al. 2019), and more convenient for laboratory manipulation. Therefore, *C. tyzzeri* may serve as a great genetic model for studying the core biological questions, and for acquiring preliminary and proof-of-concept data, prior to conducting experiments using *C. parvum* and *C. hominis*.

There are also notable reports of 2 *Cryptosporidium* gene-silencing strategies: one uses morpholino antisense oligonucleotides with success in knockdown parasite lactate dehydrogenase (*LDH*) gene expression (Witola et al. 2017; Zhang et al. 2018), and the other employs recombinant human Argonaute protein (hAgo2) pre-assembled with single-stranded RNA (ssRNA) of targeted genes (Castellanos-Gonzalez et al. 2016, 2019).

Development of *in vitro* phenotypic screening platforms

At the phenotypic screening level, *in vitro* culture of *C. parvum* in 96- or 384-well plates can be readily achieved to a satisfactory uniformity between wells by an experienced researcher. The earlier challenge for high-throughput screening or evaluating drug efficacy against cryptosporidial growth with traditional methods are either time-consuming (microscopic counting of the parasite) or have narrow linear dynamic range (e.g., ELISA or chemiluminescent assays using anti-cryptosporidial antibodies).

This obstacle has been cleared after the development and optimization of 2 platforms based on high-content imaging analysis (Bessoff et al. 2013), a luciferase assay using transgenic cryptosporidium strains (Manjunatha et al. 2017), and a qRT-PCR-based approach (Zhang and Zhu 2015, 2020). Among them, qRT-PCR assay omits a nucleic acid isolation step by directly using diluted cell lysates as templates, making it more easily adapted for use by other laboratories that lack a plate imaging facility and are unable to develop or acquire transgenic parasite strains.

Availability and limitations of animal models

In evaluating drug efficacy in animals, there is a lack of small animal models mimicking acute and chronic cryptosporidiosis with watery diarrhea, the hallmark

clinical symptom of cryptosporidiosis. Mouse neonates and several immunodeficient breeds are the most commonly used small animal models that are unable to produce diarrheal symptoms, but sufficient to assess the effect of a drug on oocyst production (Castellanos-Gonzalez et al. 2013; Guo et al. 2014, 2018; Arnold et al. 2017; Manjunatha et al. 2017; Lee et al. 2018; Baragana et al. 2019; Huang et al. 2019).

Bovine calves and gnotobiotic piglets produce watery diarrhea with experimental cryptosporidial infections, and have been developed into reproducible large animal models for evaluating anti-cryptosporidial drug efficacy (Schaefer et al. 2016; Manjunatha et al. 2017; Lee et al. 2018, 2019; Riggs and Schaefer 2020). As one of the native hosts of zoonotic *C. parvum,* drug absorption and efficacy data from bovine calves are directly relevant to treat calf cryptosporidiosis. However, it's difficult to test multiple calves in a reasonable timeframe, and data from calves may be less applicable to humans due to fundamental differences in digestive anatomy and physiology (Toutain et al. 2010; Hatton et al. 2015; Ziegler et al. 2016).

On the other hand, piglets have the advantage for infection of both *C. parvum* and *C. hominis* (Lee et al. 2018, 2019). In comparison with ruminants, drug absorption and efficacy data obtained from piglets may be more applicable to humans due to the similarity between human and porcine digestive systems (Theodos et al. 1998; Manjunatha et al. 2017; Lee et al. 2018). In addition to calves and piglets, a nonhuman primate model using pigtailed macaques (*Macaca nemestrina*) was reported in the early 1990's (Miller et al. 1990, 1991). This reproducible experimental model showed clinical symptoms as humans, and can be useful in the final stage of preclinical investigations.

Current challenges in developing therapeutics and potential solutions

As discussed above, cryptosporidial research community has confronted the unusual challenges in anti-cryptosporidial drug discovery and is making significant progress. Phenotypic screening and target-based approaches have resulted in the discovery of a number of leads displaying excellent anti-cryptosporidial efficacy *in vivo*. Some anti-cryptosporidial drug discovery and development programs are ready for (or close to) clinical investigation, while more may be in the stage of lead optimization. However, the field is still facing a final challenge in developing a clinically successful drug to treat patients experiencing severe watery diarrhea.

ED band as an extra barrier for delivery of systemic drugs

Most of the drug discovery efforts focus on systemic drugs, but the "epicellular" (i.e., intracellular but extracytoplasmic) lifestyle of parasite creates an extra burden for systemic drugs to cross ED band and reach parasite (Fig. 2). Molecular compositions and functions of parasite-host cell interface are still poorly understood, but one can assume that ED band is the major pathway for parasite to acquire nutrients from host cell cytoplasm (Leitch and He 2012). Therefore, ED band is expected to be highly "selective" in allowing molecules to pass through from host cells. This may explain that a significant number of excellent anti-cryptosporidial hits/leads identified by *in vitro* screening showed unsatisfactory efficacy *in vivo*.

A successful systemic drug must be able to pass through ED band. Data derived from mouse models can be indicative, in which the efficacy of a systemic drug would show strong correlation with pharmacokinetic parameters in plasma, rather than with luminal or fecal drug concentrations (Castellanos-Gonzalez et al. 2013; Guo et al. 2014, 2018; Arnold et al. 2017; Manjunatha et al. 2017; Lee et al. 2018; Baragana et al. 2019; Huang et al. 2019). Animal experiments are costly and time-consuming, although acute infection mouse models may be used to test more compounds with reduced costs and time (Guo et al. 2018).

Therefore, an *in vitro* assay that can conveniently evaluate the permeability of ED band for small molecules will be very helpful for prioritizing identify hits/leads for subsequent evaluation in animals. Current 2D or 3D cultivation platforms can be applied to *in vitro* evaluation of pharmacokinetic and pharmacodynamic properties of potential lead compounds prior to animal studies (Morada et al. 2016; Yarlett et al. 2020), but none of them is able to evaluate systemic drug route since drugs are directly exposed to parasite. However, it is technically achievable to grow a layer of tightly connected host cells, in which *Cryptosporidium* resides on top of host cells on one side of the layer while drugs can be applied to the other side to achieve the host-cell-to-the-parasite delivery route.

Absorption as an important factor in drug discovery against the diarrheal-causing cryptosporidiosis

Another obstacle is related to absorption of systemic drugs, as they may be "flushed out" quickly from GIT (gastrointestinal tract), giving insufficient time for absorption by hosts experiencing severe watery diarrhea. Combination of an anti-cryptosporidial drug with an anti-diarrheal medicine may improve efficacy, although anti-diarrheal medicines have a limited effect to ease cryptosporidial-induced diarrheal symptoms (Checkley et al. 2015).

Novel pharmacological modifications and formulations may be employed to increase drug absorption in individuals experiencing severe diarrhea, such as by enhancing

drug's mucoadhesion property (Pridgen et al. 2015; Purohit et al. 2018), and/or by exploring the enterohepatic recycling pathway to increase gastrointestinal exposure and bioavailability (Xia et al. 2012; Dai et al. 2015).

Evaluation of drug absorption and anti-cryptosporidial efficacy in animals is also a challenge, because these properties cannot be fully assessed in current mouse models that exhibit no diarrheal symptoms, and routine evaluation of a large number of drugs in neonatal calf and gnotobiotic piglet models are impractical. Therefore, the development of more convenient piglet models (vs. gnotobiotic piglets) can be very helpful to overcome the bottleneck in preclinical evaluation of anti-cryptosporidial drugs.

Non-systemic drugs as an alternative strategy

Another approach is to develop "non-systemic" drugs to more directly act on the epicellular parasite in GIT. It is known that some compounds cannot be well absorbed, or unable to effectively pass ED band to act on parasite, but gastrointestinal exposure is critical to their anti-cryptosporidial activity (e.g., paromomycin and some bumped kinase inhibitors) (Arnold et al. 2017).

More recently, a glycolipopeptide occidiofungin featured by poor absorbability and GIT retainment was found to be highly efficacious on *C. parvum in vitro* with limited cytotoxicity (Ma et al. 2020). These observations suggest that non-systemic drugs may be an effective alternative to develop systemic drugs. However, non-systemic drugs would still face the same challenge of being flushed out by diarrhea, which may be resolved by increasing the mucoadhesive property and/or in combination of anti-diarrheal medicines to increase their retention in host GIT.

Conclusions

Cryptosporidiosis is an important zoonotic disease for which fully effective treatments are unavailable. The epicellular (intracellular, but extra-cytoplasmic) parasitic lifestyle makes the diarrheal pathogen cryptosporidium different from other enteric coccidia that reside within host cell cytosol, and creates unique challenges in developing therapeutics. Systemic drugs need to be not only absorbed rapidly by GIT of humans and animals that typically experiencing severe watery diarrhea, but also to effectively pass through the selective ED band to act on parasite. It would be helpful to develop an *in vitro* assay to effectively screen hits/leads for their permeability over ED band.

Non-systemic drugs may be an effective alternative direction, as they only need to cross PVM to act on parasite. For non-systemic drugs, the ability to cross PVM is not a major concern for hits/leads that already show satisfactory efficacy *in vitro*. Challenge is their retainment in GIT of individuals experiencing diarrhea. For both systemic and non-systemic drugs, improving mucoadhesive property by formulation or chemical modification would be helpful to absorption for systemic drugs and to retainment for non-systemic drugs, to achieve significant efficacy in humans.

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Authors' contributions

All authors have read and approved the final version of the manuscript.

Declarations

Competing interests

The authors declare that they have no competing interests.

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