From the Institute of Parasitology
Faculty of Veterinary Medicine, Leipzig University

**Studies into the suitability of the cell-penetrating peptide
octaarginine as a transmembrane vehicle for DNA transfection of
Cryptosporidium parvum and to improve the antiprotozoan
efficacy of Nitazoxanide**

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Dedicated to my HOMELAND and my FAMILY

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List of abbreviations

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Caco-2	Heterogenous human epithelial colorectal adenocarcinoma
COLO-680N	Oesophageal squamous-cell carcinoma
COWP	<i>Cryptosporidium</i> oocysts wall protein
CPP	Cell-penetrating peptide
CPPs	Cell-penetrating peptides
DNA	deoxyribonucleic acid
DFA	Direct fluorescence assay
DMSO	Dimethy sulfoxide
dpi	Day post infection
DVG	Deutsch Veterinärmedizinische Gesellschaft
EIA	Enzymimmuno assay
FDA	Food and Drug Administration
GP60	60-kDA glycoprotein gene
HCT-8	Human ileocecal adenocarcinoma
HFK	Human Foreskin Keratinocytes
HIV	Human immunodeficiency virus
HS	Heparin sulfate
Hsp	Heat shock protein
IC50	The half maximal inhibitory concentration
ICZN	Zoological Nomenclature
IFA	Immunofluorescence assay
MDBK	Madin-Darby bovine kidney
MDCK	Madin-Darby canine kidney
MTT	3-(4,5- dimethylthiazole-2-yl) 2-5- diphenyltetrazolium bromide
MZN	Modified Ziehl-Neelsen
NaOCl	Sodium hypochlorite
NTZ	Nitazoxanide

List of abbreviations

NTZ-R8	Nitazoxanide-octaarginine
OA	Oligoctaarginine
PEI	Polyethylenimine
PFOR	Pyruvate ferredoxin oxireductase
PGI	Parasite growth inhibition
PRM	Paromomycin
PV	Parasitophorous vacuole
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RL 95-2	Human endometrial carcinoma
RT-PCR	Reverse transcriptase polymerase chain reaction
SGLT	Sodium-glucose cotransporter
STE	Short-time excysted
TAT	Transcription- transactivatin

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1. Introduction

Cryptosporidium is a small protozoan parasite which causes gastroenteritis in both animals and humans (OLSON et al. 2004). Among the *Cryptosporidium* genus, *Cryptosporidium hominis* and *Cryptosporidium parvum* are the two most important species infecting humans (XIAO and RYAN 2004, RYAN et al. 2016). While *C. hominis* seems to infect exclusively humans, *C. parvum* is zoonotic (FELTUS et al. 2006). *C. parvum* is a most important pathogen related to watery diarrhea in ruminant livestock, especially in neonate calves. It causes significant economic loss in animal husbandry (THOMSON et al. 2017). In humans, cryptosporidiosis can become life-threatening in immunocompromised individuals such as AIDS patients, or malnourished children (VENTURA et al. 1997, HUNTER and NICHOLS 2002). A recent global study covering all enteric diseases has classified cryptosporidiosis as the second most relevant pathogen, behind rotavirus, responsible for diarrhea in children (KOTLOFF et al. 2012). Importantly, *Cryptosporidium* has also been associated with outbreaks of water-borne diseases worldwide in industrialized countries such as South Korea (MOON et al. 2013), Australia (WALDRON et al. 2011), and the United States of America (GHARPURE et al. 2019). Therefore, *Cryptosporidium* has not only a tremendous impact on veterinary public health but has also to be considered in the context of the “One Health” concept.

Until now, there is no vaccine available for cryptosporidiosis. Nitazoxanide (NTZ) is the only drug approved to date by the US Food and Drug Administration (FDA) for treatment of cryptosporidiosis in humans but shows only a limited efficacy (SCHNYDER et al. 2009), especially in the most vulnerable target groups (CABADA and WHITE. 2010, MEAD and ARROWOOD 2014). For most anti-cryptosporidial drugs, the transition from *in vitro* experimental data to *in vivo* application represents an insuperable challenge, e.g. due to poor system bioavailability and to toxicity of drug candidates (LOVE et al. 2017, BHALCHANDRA et al. 2018).

Therefore, the goal of the current study is to evaluate the delivery of therapeutic compounds such as NTZ across the multiple membrane layers that protect the intracellular pathogen in order to improve bioavailability, thus increasing the efficacy and reducing dosage of potentially toxic anti-cryptosporidial compounds. Cell-penetrating peptides (CPPs) are promising candidates in drug delivery (KRISTENSEN et al. 2016). CPPs in general are short polycationic peptides (7-30 residues) which have been applied in various studies to improve delivery of small molecules including DNA, RNA, and certain pharmaceuticals into cells (BECHARA and SAGAN 2013, DINCA et al. 2016). CPPs cross the blood-brain barrier and have been demonstrated to penetrate through biological membranes into cytoplasmic and nuclear compartments (STALMANS et al. 2015). Interestingly, CPPs do not lose their properties when they are attached to cargos of different structure and size. The potential of octaarginine to greatly enhance efficacy of drugs has been shown for other intracellular parasites such as *Plasmodium* (SPARR et al. 2013). Therefore, the objectives of the current work were to investigate the potential of the octaarginine

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as delivery vehicle for small molecules into *Cryptosporidium*. Two specific objectives were established. Firstly, the application of octaarginine to deliver plasmid DNA for transfection purpose was developed (PUBLICATION 1). Secondly, the coupling of octaarginine to NTZ to improve its efficacy was evaluated *in vitro* and *in vivo* (MANUSCRIPT 2)

2. Literature review

2.1 Biology and taxonomic status

Cryptosporidium was first described and classified as a protozoon of uncertain taxonomic status by Ernest Edward Tyzzer based on its asexual and sexual stages (TYZZER 1907). Later, ultrastructural studies on *Cryptosporidium* found that this genus possesses a special attachment organelle, which is considered a key feature typical for the genus *Cryptosporidium*. Morphology, particularly of oocysts, development biology, and host specificity are other classical features to allocate cryptosporidia to a certain species, complying with the international code of Zoological Nomenclature (ICZN) rules. With the achievements of molecular biology, gene sequence information has been widely applied in defining new species of *Cryptosporidium*. Although *Cryptosporidium* is still allocated to the phylum Apicomplexa, this genus distinctly differs from other members of this phylum e.g. by lacking an apicoplast and any trace of the apicoplast genome, and mitochondrion (ZHU et al. 2000). Moreover, motility and the invasion process differ from other apicomplexa (WETZEL et al. 2005). Meanwhile, based on microscopy, biochemical and genomic data, *Cryptosporidium* is officially classified into the class of *Gregarinomorphea*, subclass *Cryptogregaria* (RYAN et al. 2016) as follows:.

Empire: Eukaryota
 Kingdom: Protozoan
 Phylum: Apicomplexa
 Class: Gregarinomorphea
 Subclass: Cryptogregaria
 Order: Eucoccidiorida
 Suborder: Eimerinoria
 Family: Cryptosporidiidae
 Genus: *Cryptosporidium* Tyzzer, 1970

2.1.1 Life cycle of *Cryptosporidium*

Cryptosporidium infection is initiated by ingestion of sporulated oocysts from the environment, e.g. food or water contaminated with feces that are excreted by an infected host (LEITCH and HE 2011). Unlike oocysts of typical coccidia, *Cryptosporidium* oocysts are already sporulated and thus fully infective upon excretion. Under the activity of digestive enzymes such as those produced by the pancreas and bile salts, the excystation process occurs in the small intestine of the host. Four banana shaped sporozoites of 3-5 μm length are liberated from each oocyst and invade the brush border of enterocytes but do not penetrate to the host cell membrane as other apicomplexa do. In contrast, *Cryptosporidium* sporozoites trigger the host cell membrane to embrace the parasite and to form a parasitophorous vacuole

(PV) around the parasites. The PV thus remains extracytoplasmatic although the parasite is located intracellular since it is fully surrounded by host cell membrane.

A particular feature of the PV is an actin disc that is formed at the basal area underneath the attached parasite and probably serves to transport nutrients from the cytoplasm to the PV (“feeder organelle”) during the internalization process, the sporozoites become spherical and are now called trophozoites (1-2 μm) (BOROWSKI et al. 2010). The trophozoites start to replicate by transforming into meronts. During the asexual replication in type I meronts 6-8 merozoites develop in each meront. Merozoites have a similar structure as sporozoites with a small size (0.4 $\times$ 1.0 μm). Type I merozoites infect adjacent epithelial cells, developing again into trophozoites and then type II meronts. Merozoites produced by type II meronts initiate the sexual replication. During this phase, macrogamonts (4 x 6 μm) and microgamonts (2 x 2 μm) are formed (LEITCH and HE 2011). Microgametes are released from microgamonts and fertilize macrogamonts to form diploid zygotes that transform into oocysts. During sporulation of the oocysts meiosis occurs resulting in 4 haploid sporozoites per oocyst. A firm oocyst wall is formed. Approximately 80% of oocysts produced in the small intestine are considered thick walled and the others are thin-walled (FAYER 2008). Thin-walled oocysts are described to be responsible for autoinfection whereas the thick-walled oocysts are released with the feces to infect new hosts and have the capacity to survive for a long time in the environment. Through the life cycle of *Cryptosporidium*, autoinfection occurs at two stages: first by recycling of merozoites of type I meronts and secondly through sporozoites excysted from thin-walled oocysts (Figure 1)

Figure 1: Diagrammatic representation of the life cycle of *Cryptosporidium* (Image adopted from LENDNER AND DAUGSCHIES 2014)

2.1.2 The formation of the parasitophorous vacuole (PV)

The invasion process starts when sporozoites attach to the luminal surface of an epithelial enterocyte. At that moment, contents of rhoptries, micronemes and dense granules are excreted at the anterior tip of the sporozoite (FAYER 2008). Apical proteins have been demonstrated to be essential in the invasion process (CHEN et al. 2004). The engulfing of parasite and the establishment of the PV are related to swelling of the host cell microvilli as aquaporin I and the sodium-glucose cotransporter (SGLTI) are recruited to the host cell-parasite interface (CHEN et al. 2005). As a result, the PV is formed as a bubble-like structure induced by both parasite and host cell. Moreover, an electron dense band subsequently matures into a unique structure, the so-called feeder organelle that gives the parasite access to nutrient of the host cell for its own development (CHEN et al. 2005, ZHU 2008). It is assumed that the lack of efficient anti-cryptosporidial drugs reflects the fact that intracellular stages are protected by the PV and not easily accessible to drug treatment (MIYAMOTO and ECKMANN 2015)

2.1.3 Metabolic pathways of *C. parvum*

Cryptosporidium parvum has a genome size of 9.1 Mb (ABRAHAMSEN et al. 2004) which is thus much smaller than that of other apicomplexan parasites, such as *Plasmodium falciparum* (22.9 Mb) or

Toxoplasma gondii (80 Mb) (GARDNER et al. 2002). *C. parvum* lacks mitochondria, the apicoplast, and genes coding for Krebs cycle, but it still possesses genes which encode for mitochondrial proteins, particularly TOM40 and TIM17, and chaperons like HSP70 and HSP80. However, *C. parvum* has lost most *de novo* synthesis capacities to produce fatty acids, amino acids and nucleosides, and thus this parasite almost completely depends on the nutrient sources provided by the host cell (ZHU 2008).

2.2 Epidemiology

2.2.1 Cryptosporidiosis in humans

Cryptosporidium infection is characterized by self-limiting diarrhea, nausea, vomiting and abdominal pain. Immunocompetent people can recover by themselves after 2-3 weeks (LEITCH and HE 2011, RAJ MAINALI et al. 2013). However, the infection can cause mortality, malabsorption and wasting in immunocompromised people such as HIV patients or malnourished children. In developing countries, cryptosporidiosis is the second most frequent diarrhea pathogen associated with mortality in children, just after Rotavirus (KOTLOFF et al. 2013). Annually, there are more than 200000 children worldwide who die because of *Cryptosporidium* infection (NASRIN et al. 2013). Most of these cases are associated to malnutrition and poor hygiene and unfavorable living conditions in developing countries in Asia and Africa (MONDAL et al. 2009). *Cryptosporidium* infection in early childhood has a permanent influence on child growth, which is associated with growth retardation, malnutrition, cognitive deficits and impaired immune response (MØLBAK et al. 1997, RYAN et al. 2016). In Germany, human cryptosporidiosis cases have been reported annually to range from 900 to 1400 cases between 2008 and 2012 (GERTLER et al. 2015).

2.2.2 Cryptosporidiosis in animals

Cryptosporidium is a common parasite which infects both domestic and wild animals worldwide. Ruminants are common hosts for *Cryptosporidium* infection, particularly neonate animals. It has been shown in epidemiological reports, that the prevalence and severity of cryptosporidiosis is related to the age of animals, and young animals generally present higher prevalence than adults (SANTÍN et al. 2004, MADDOX-HYTTEL et al. 2006, FAYER et al. 2007). Cryptosporidiosis in neonate cattle may lead to profuse watery diarrhea, dehydration, weight loss, and reduced appetite, and in some cases death can occur (THOMSON et al. 2017). *C. parvum* occurs worldwide and its prevalence in pre-weaned calves is estimated to range from 3.4 to 96.6 % (THOMSON et al. 2017), in Europe prevalence from 20-40% has been reported based on evaluation of routine diagnostic records (JOACHIM et al. 2003). Recent epidemiological studies revealed much higher prevalence in Germany and it was supposed that almost every pre-weaned calf will attract infection sooner or later (HOLZHAUSEN et al. 2019). In cattle four

Cryptosporidium species, *C. parvum*, *C. bovis*, *C. andersoni* and *C. ryanae*, have been reported (XIAO and PENG 2008). Among them, only *C. parvum* is considered to be pathogenic and zoonotic.

2.2.3 Transmission pathway and water-borne disease

Cryptosporidium oocysts are transmitted among hosts via the fecal-oral route, which includes a direct and an indirect pathway. The direct transmission occurs during the contact with feces of infected animals, while the indirect transmission occurs by ingestion of contaminated water or food. *Cryptosporidium* is responsible for large waterborne outbreaks in developed countries (KARANIS et al. 2007). In a recent study, it was stated that *Cryptosporidium* is responsible for more than 8 million cases of foodborne illness every year (RYAN et al. 2018). Rainfall and water flooding contribute to the spread of pathogens in general, including *Cryptosporidium*. For instance, an outbreak occurred in Halle, Germany, where 24 cases of cryptosporidiosis were documented 6 weeks after the river Saale overflowed the floodplains and parts of the city (GERTLER et al. 2015). Most attention was attracted to waterborne cryptosporidiosis following a large outbreak in Milwaukee that was reported to have affected over 400000 people (KENZIE et al. 1994). This outbreak was obviously not zoonotic since *C. hominis* was identified as the causative agent (SULAIMAN et al. 2001).

2.2.4 Detection and diagnosis

There are various methods to detect *Cryptosporidium* in fecal samples such as microscopy (with or without staining of samples), immunofluorescent labeling and genetic evaluation.

Microscopy

Modified Ziehl-Neelsen (MZN) staining has been proposed as the golden standard in *Cryptosporidium* diagnosis. *Cryptosporidium* oocysts are stained by MZN red on a blue background. However, it requires technical expertise in interpretation when just few oocysts are present since the samples may contain *Cryptosporidium* oocyst-like bodies such as fungal spores or, in human feces, oocysts of *Cyclospora*. The negative staining with strong carbol fuchsin (HEINE staining) is frequently used to screen samples for *Cryptosporidium* oocysts because it is fast and inexpensive. However, the sensitivity is lower than reported for MZN (POTTERS and VAN ESBROECK 2010).

Immunofluorescence assay

Direct or indirect immunofluorescence assays (DFA or IFA) provide higher sensitivity than microscopy. However, these methods may be not affordable depending on the economic situation. Moreover, they require advanced technological equipment as a UV microscope for visualizing (CHALMERS and KATZER 2013)

Enzyme immunoassay

Commercial kits are often based on enzymeimmuno assay (EIA) methodology to detect *Cryptosporidium* antigen in stool samples. Attractive features of these kits are that they are simple to use, time saving and do not demand any special equipment like laboratory microscopes. However, they are normally quite expensive and sensitivity and specificity may vary ranging from 66.3% to 100% and 93%-100%, respectively.

(<https://www.cdc.gov/dpdx/diagnosticprocedures/stool/antigendetection.html>)

Molecular techniques

Molecular techniques have been widely applied to detect *Cryptosporidium* in a variety of types of samples such as feces, tissue, water or foods. Different house-keeping genes can be targeted to detect *Cryptosporidium* such as 18S rRNA, heat shock protein (Hsp70), *Cryptosporidium* oocyst wall protein (COWP) (XIAO and RYAN 2004). The benefits of the molecular technique are not only high sensitivity and specificity for *Cryptosporidium* but also that they have the capacity to differentiate the various species of this parasite, allowing to distinguish *Cryptosporidium* species in clinical samples or in the environment (CHALMERS and KATZER 2013). The 60-kDA glycoprotein gene (GP60) is the most popular marker and is used for subgenotyping of *C. parvum* or *C. hominis* (WIELINGE et al. 2008, ZAHEDI et al. 2016). GP60 subgenotyping enables epidemiologic evaluation and comparison of the distribution of subtypes of *Cryptosporidium* spp. among different studies, countries and hosts. Real time-PCR is also a commonly applied and useful molecular method. Depending on the selection of the most suitable primers and probes, RT-PCR is much more sensitive than conventional methods and can detect as few as 2 oocysts in a sample (HADFIELD et al. 2011). In addition, RT-PCR may be used to quantify the amount of parasite DNA in samples while conventional PCR does not allow quantification.

2.3 Therapeutics

2.3.1 Halofuginone lactate

Halofuginone lactate is approved for cryptosporidiosis treatment and prophylaxis in neonate calves in Europe. This substance is mainly active on the extracellular stages of *Cryptosporidium parvum*, i.e. sporozoites and merozoites. It prevents the intracellular invasion by *C. parvum* and the forming of oocysts (JARVIE et al. 2005). Therefore, it significantly decreases oocyst excretion via feces and clinical symptoms related to enteritis (JOACHIM et al. 2003, BOROWSKI et al. 2010, KEIDEL and DAUGSCHIES 2013). By strategic application of this drug, environmental contamination by oocysts and thus infection pressure on other calves may be distinctly reduced. However, the precise mechanism how halofuginone affects the lifecycle of *C. parvum* is not well understood (THOMSON et al. 2017)

Halofuginone is used in suckling calves by oral administration once per day on 7 consecutive days (LEFAY et al. 2001, JARVIE et al. 2005). The drug should be used with great care according to manufacturer recommendations to avoid toxic effects (JARVIE et al. 2005)

2.3.2 Paromomycin (PRM)

PRM belongs to the aminoglycoside antibiotic group and is produced by *Streptomyces rimosus*. It has been applied for *Cryptosporidium* treatment both *in vitro* and *in vivo* (mouse model and in human) (STOCKDALE 2008). However, the mechanism how PRM inhibits *Cryptosporidium* infection is unknown. It has been supposed that during the initial asexual development of *Cryptosporidium* the level of protein synthesis is high and that PRM causes translation of mRNA thus inhibiting parasite protein synthesis (SHARMA et al. 2014). *In vitro*, PMR applied at a dose of 6 mg/ml reduced viability of *Cryptosporidium* oocysts and inhibited growth and invasion (SHARMA et al. 2014). PRM was documented to inhibit intracellular stages of *Cryptosporidium* in cell culture (GRIFFITHS et al. 1998), in tissue cultures and *in vivo* in immunocompromised mice (VINAYAK et al. 2015). PRM has been recommended for cryptosporidiosis prophylaxis in calves at a dose of 100 mg/kg for 7 days by oral administration. This treatment reduced oocyst shedding and the number of diarrhea days (AYDOGDU et al. 2018). PRM showed only low efficacy in cryptosporidiosis in patients with AIDS. It was found that there was no difference in improvement between the PRM treated patient group and placebo patient group (WHITE et al. 1994, HEWITT et al. 2000).

2.3.3 Nitazoxanide (NTZ)

NTZ is a thiazolide antiparasitic agent that affects a broad spectrum of protozoa and helminthes. Studies demonstrated the efficiency of NTZ in *Cryptosporidium* and *Giardia* treatment *in vitro* and *in vivo* (FOX and SARAVOLATZ 2005, RAJ MAINALI et al. 2013). Although the mechanism of NTZ in inhibiting *Cryptosporidium* is not completely understood, it has been attributed to a capacity of NTZ to inhibit pyruvate-ferredoxin oxidoreductase (PFOR), an enzyme essential to anaerobic energy metabolism. NTZ was demonstrated to inhibit the growth of sporozoites of *C. parvum*. Moreover, in combination with antibiotics such as azithromycine or rifampin NTZ displayed better efficacy of treatment as compared to application of NTZ alone (GIACOMETTI et al. 2000). In MDBK cells NTZ at the concentration of 10 µg/ml reduced parasite growth by more than 90% (THEODOS et al. 1998). Later, the efficacy of NTZ in *C. parvum* isolates (IOWA and KSU1) was evaluated by using real time PCR, and the results showed that the growth of parasites was almost completely inhibited by NTZ at a concentration of 12.5 µg/ml (CAI et al. 2005), confirming the previous studies. In another study, NTZ (25µg/ml) reduced oocyst viability, invasion and growth of *C. parvum* in MDCK cells by 95.1%, 98.1% and 99.1%, respectively (SHARMA et al. 2014), and was thus much more efficient than PRM.

Up to now, NTZ is the only drug approved by FDA for *Cryptosporidium* treatment in humans. In one study, adults and children infected with *Cryptosporidium* were treated with NTZ at a dose of 100 mg, 200 mg and 500 mg, corresponding to age groups 1-3 years, 4-11 years, and over 12 years old, respectively. After treatment, the oocyst shedding in stool and diarrhea were reduced significantly as compared to a placebo group (ROSSIGNOL et al. 2001). Application of NTZ on 3 consecutive days (100 mg twice per day) was recorded to resolve symptoms of disease and to prevent oocyst excretion in 52 % of malnourished children in Zambia that were serologically HIV negative but nonetheless suffered from chronic cryptosporidiosis (AMADI et al. 2002). However, controversial data exist on the efficacy of NTZ in the control of cryptosporidiosis in children and HIV patients. Placebo controlled trials proved that NTZ showed limited efficacy in HIV patients (CABADA and WHITE 2010, MEAD and ARROWOOD 2014). Although NTZ displayed little capacity to inhibit *Cryptosporidium* in immunocompromised patients, particularly HIV patients, it is the only licensed drug for cryptosporidiosis treatment in humans. Attempts to develop novel pharmaceuticals against *Cryptosporidium* have been evaluated *in vitro* and *in vivo*, such as oleylphosphocholine (SONZOGNI-DESAUTELS et al. 2015), pyrazolopyridines inhibiting Cp PI(4) (MANJUNATHA et al. 2017), and benzoxaborole (LUNDE et al. 2019). In initial studies, those drugs showed better efficacy than NTZ. However, further clinical research in humans is necessary to get these compounds registered for treatment.

2.4 Cell culture models

A variety of cell lines has been established for cultivating *Cryptosporidium*. Such models have been extensively used for basic research, drug screening, propagation as well as analysis of host-parasite interaction. Mostly, the human ileocecal adenocarcinoma cell line (HCT-8) is preferred for *in vitro* studies, however, other cell types such as Madin-Darby bovine kidney cells (MDBK), Madin-Darby canine kidney cells (MDCK), heterogenous human epithelial colorectal adenocarcinoma cells (Caco-2), cells derived from human endometrial carcinoma (RL 95-2), or African green monkey kidney cells (BS-C-1) (BONES et al. 2019) have been also used in *Cryptosporidium* research. Although cell lines are powerful tools for *in vitro* studies about *Cryptosporidium*, it is obvious that they do not allow completion of the whole parasite life cycle and are not suited for continuous *Cryptosporidium* propagation. Although it has been reported that oocysts of *C. parvum* can be recovered in low numbers from infected HCT-8 cells, such observations could not be confirmed (GIROUARD et al. 2006).

In a recently published study, COLO-680N (oesophageal squamous-cell carcinoma) cells were reported to be suited for propagation of *C. parvum* oocysts (MILLER et al. 2018). However, these results could not be reproduced by others (ZHENG et al. unpublished). Attempts to produce *Cryptosporidium* oocysts in axenic culture (HIJJAWI et al. 2010, ALDEYARBI and KARANIS 2016) or aquatic biofilm (KOH

et al. 2013) were published. Although the different stages of *Cryptosporidium* (asexual and sexual stages) have been confirmed by conventional microscopy and ultrastructure analysis by TEM in such free-cell cultures, the major limitation is failure to achieve long-term propagation and low yields of oocysts (KARANIS 2018). Altogether, all efforts to produce *C. parvum* oocysts *in vitro* in suitably high numbers have failed so far, and therefore animal models cannot be completely replaced by cell culture in *Cryptosporidium* research by now.

2.5 *In vivo* models

Animal models such as rodents, gnotobiotic piglets (THEODOS et al. 1998, LEE et al. 2019), and calves (KEIDEL and DAUGSCHIES 2013, MANJUNATHA et al. 2017) have been used for *Cryptosporidium* research. They are indispensable for pharmacological investigations, particularly screening of drugs and evaluation of efficiency based on clinical parameters. Particularly immunodeficient breeds (e.g. SCID mice) (TZIPORI et al. 1995), neonate mice (DOWNEY et al. 2008) and INF- γ knockout mice (SONZOGNI-DESAUTELS et al. 2015) proved to be suitable models for *in vivo* research. INF- γ plays an important role in self-limiting *Cryptosporidium* infection in humans (MEAD 2014) by directly inducing enterocyte resistance against *Cryptosporidium* infection (POLLOK et al. 2001). It has been supposed that knocking out of the gene encoding for IFN- γ in mice can at least partly mimic the lack of immune response in immunocompromised human patients who particularly suffer from *Cryptosporidium* infection (MANJUNATHA et al. 2017). A major advantage of INF- γ knockout mice is that they rapidly display clinical symptoms, such as wasting. The lesions observed in the entire small intestine of infected mice resemble those caused by *Cryptosporidium* infection in immunocompromised humans (GRIFFITHS et al. 1998). Hence, the INF- γ knockout mouse model is the preferred rodent model and used by many research groups.

2.6 Vaccination

Efforts have been undertaken to identify and characterize vaccine candidates both *in vitro* and *in vivo* (MEAD, 2014). For instance, inoculation with gamma-irradiated *C. parvum* oocysts prevented clinical symptoms and reduced oocyst shedding in dairy calves (JENKINS et al. 2004). Surface antigens of *Cryptosporidium* sporozoites such as glycoprotein Cp15 and P23 are possibly suitable candidates to develop a protective vaccine (FEREIG et al. 2018). These antigens provided promising results in goats, BALB/c mice, C57BL/6 mice, and Interleukin-12 knockout mice by reducing the number of oocysts excreted and clinical symptoms (HE et al. 2005). However, although the process in research on vaccines is continuous, no vaccine is available up to date.

2.7 Control strategies

2.7.1 In human

Cryptosporidium is transmitted by zoonotic and non-zoonotic pathways (waterborne, foodborne and nosocomial transmission) (VANATHY et al. 2017). *Cryptosporidium* oocysts are resistant to harsh environmental conditions and may survive e.g. at low temperature of -10 °C for 1 week or 4 days in dry feces. They are resistant to most common disinfectants such as chlorine-based products at any concentration within the concentration range applicable for drinking water treatment (FAYER 1995, CHALMERS and GILES 2010).

Noticeably, outbreaks of cryptosporidiosis in humans are frequently related to waterborne transmission. Therefore, accessing to clean drinking water is crucial to avoid human cryptosporidiosis. Options to control the quality of drinking water have been evaluated such as using UV light or filtration before supply of drinking water to households. If no oocyst-free drinking water is accessible, boiling is a proper measure to inactivate oocysts. Surface water used for recreation or swimming pools may also serve as a source of infection. Disinfection of swimming pools by hyperchlorinate is considered an appropriate measure. People with diarrhea are strongly recommended to avoid swimming to protect their own health and community (GHARPURE et al. 2019). Altogether, good hygiene practices are a crucial key to prevention of transmission of cryptosporidiosis in human populations.

Remarkably, ingestion of only nine oocysts of *C. parvum* can induce infection in humans (OKHUYSEN et al. 1999) while one infected calf may release approximately 10^{10} oocysts (MOORE et al. 2003). *Cryptosporidium* infections in human populations may be zoonotic (in particular *C. parvum*) or non-zoonotic (*C. hominis*) and thus cannot successfully be controlled by only applying control measures in humans. It is estimated that 15% of *Cryptosporidium* infection of humans are linked to direct or indirect contact to infected animals (GHARPURE et al. 2019). To reduce risk of zoonotic transmission it is necessary to reduce as much as possible the excretion of infectious oocysts by infected animals and to implement the best possible hygienic measures in animal housings.

2.7.2 In animals

Effective strategies include a combination of proper hygiene, prophylactic treatment, and best possible management of neonate animals (HARP and GOFF 1998, THOMSON et al. 2017). Environmental contamination with oocysts is the main source of *Cryptosporidium* infection of calves. Keeping good hygiene practices should consider intensive cleaning of surfaces, pathogen-free drinking water and troughs. In farms where calves suffer from diarrhea caused by *Cryptosporidium*, it is crucial to apply steam-cleaning or hot water for cleaning, followed by drying the surfaces (HARP and GOFF 1998)

because *Cryptosporidium* oocysts are susceptible to high temperature and desiccation (FAYER 2004). For decontamination of well-cleaned, dry surfaces commercial disinfectant products may be used. A collection of suitable products is listed by the German Veterinary Society (Deutsche Veterinärmedizinische Gesellschaft, DVG) (column 8b, protozoan parasites). This list is continuously updated and freely accessible under “www.desinfektion-dvg.de”. Keeping newborn calves in well cleaned single crates or igloos will reduce exposure to oocysts. Sufficient colostrum application is essential. Observation of young calves for signs of gastroenteritis and coproscopical examination in case of a suspected outbreak of cryptosporidiosis should be considered by farmers, as well as protection of calves from other enteropathogens that may aggravate the disease (GÖHRING et al. 2014). If it is necessary, treatment with halofuginone has to be initiated as early as possible (within 24 h after birth or first observation of disease) and correctly performed (100 µg/kg BW daily over 7 days) (SHAHIDUZZAMAN and DAUGSCHIES 2012).

2.8. Cell-penetrating peptides (CPPs)

CPPs are short cationic peptides that consist of less than 30 amino acid residues. The two first discovered CPPs are TAT and penetratin. TAT is the transcription-transactivatin (TAT) protein of the human immunodeficiency virus (HIV), while penetratin is derived from the *Drosophila antennapedia* homeodomain. FRANKEL and PABO (1988) demonstrated that TAT could penetrate and translocate into the cell nucleus and JOLIOT et al (1991) showed that penetratin could be internalized by neuronal cells. CPPs possess the capacity to evoke the process of translocation across the cell membrane, mitochondrial and nucleus membranes and cross through the blood-brain barrier (LINDGREN et al. 2000, MÄE and LANGE 2006). They are able to enter prokaryotic and eukaryotic cells without altering cellular integrity. Remarkably, they have the ability to deliver a variety of compounds (cargoes), particularly small molecules, nucleic acids, proteins, counting imaging agents, or drug molecules, fluorescent probes, metal-binding ligands, etc. into cells (BORRELLI et al. 2018)

2.8.1 Classification of cell-penetrating peptides

CPPs can be classified based on different criteria such as the origin (natural or synthetic products), the chemical structure, or the mechanism of how they penetrate into the cell. In table 1, CPPs classification according to structure is presented. CPPs may be cationic, amphipathic or anionic (HABAULT and POYET 2019).

Table 1: Classification of CPPs according to HABAULT and POYET (2019)

Peptide	Sequence	Type	Length	Origin
Antennapedia Penetratin (43–58)	RQIKIWFQNRRMKW KK	Cationic and amphipathic	16	Protein-derived
HIV-1 TAT protein (48–60)	GRKKRRQRRRPPQ	Cationic	13	Protein-derived
pVEC Cadherin (615– 632)	LLIILRRRIRKQAHAAH SK	Amphipathic	18	Protein-derived
Transportan Galanine/Mastoparan	GWTLNSAGYLLGKI NLKAL AALAKKIL	Amphipathic	27	Chimeric
MPG HIV-gp41/SV40 T-antigen	GALFLGFLGAAGST MGAWSQPKKKRKV	Amphipathic	27	Chimeric
Pep-1 HIV-reverse transcriptase/SV40 T- antigen	KETWWETWWTEWS QPKKKRKV	Amphipathic	21	Chimeric
Polyarginines	R(n); $6 < n < 12$	Cationic	6-12	Synthetic
MAP	KLALKLALKALKAA LKLA	Amphipathic	18	Synthetic

Table 1 continued

R6W3	RRWWRRWRR	Cationic	9	Protein-derived
NLS	CGYGPKKKRKVGG	Cationic	13	Protein-derived
8-lysines	KKKKKKKK	Cationic	8	Synthetic
ARF (1-22)	MVRRFLVTLRIRRAC GPPRVRV	Amphipathic	22	Protein-derived
Azurin-p28	LSTAADMQGVVTDG MASGLDKDYLPDD	Anionic	28	Protein-derived

2.8.2 Mechanism of cellular uptake of CPPs

Until now, the exact mechanism of how specific cell-penetrating peptide internalize into cells is not completely understood. Most CPPs utilize two or multiple pathways for translocation into the host cells (MADANI et al. 2011) which depends not only on the characteristics of the respective CPPs but also on experimental conditions. In general, there are two main mechanisms: direct penetration and endocytosis as visualized in Figure 2.

Figure 2: Schematic presentation of cellular uptake mechanism related to CPPs and their cargo (modified drawing from LAYEK 2015)

Direct penetration is one pathway leading to membrane translocation. It is fast and energy-independent. Various models have been proposed to explain the related mechanisms, such as the carpet-model, pore-forming, inverted micelles or the membrane thinning model (MADANI et al. 2011). The first step of these mechanisms is mostly based on the interaction of positively charged CPPs with the negatively charged components of the cell membrane, e.g. bilayer phospholipids and heparin sulfate (HS) (MADANI et al. 2011, KAUFFMAN et al. 2015). This process contributes to permanent or temporary destabilization of the cell membrane, which facilitates the folding of peptides on the lipid membrane. There are many factors such as the concentration, amino acid sequences which influence the internalization process (KALAFATOVIC and GIRALT 2017). Normally, the direct penetration happens with high concentration of CPPs or when amphipathic CPPs such as transportant analogues and MPG (a fusion between a hydrophobic domain from HIV gp41 and hydrophilic domain from the nuclear localization sequence of SV40 T-antigen are applied (DUCHARDT et al. 2007).

The mechanism of direct translocation of penetratin has been explained mainly on the basis of the model of the “inverted micelle” (DEROSSI et al. 1998). Electrostatic interaction between CPPs and negatively charged compounds of the cell membrane result in local disorder of the phospholipid bilayer, which

forms inverted hexagonal structures. Peptides are encapsulated in the hydrophilic interior environment of the micelle. Inversion of the micelle at the inner layer of the membrane leads to peptide release into the cytoplasm (KAWAMOTO et al. 2011).

Endocytosis by phagocytosis or pinocytosis is an energy-dependent process. Phagocytosis is also known as “cell eating” and occurs in scavenger cells such as macrophages, neutrophils, and leukocytes. This process leads to engulfment of solid particles such as bacteria or protozoan parasites that are enclosed into phagosome vesicles. Phagosomes fuse with lysosomes to form phagolysosomes (CLEAL et al. 2013). In contrast, pinocytosis involves engulfment of liquids or solutes and is referred as “cell drinking”. Pinocytosis is important for cellular homeostasis control and occurs in every cell type. Pinocytosis is classified as macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis or clathrin- and caveolin-independent cytosol (JONES 2007, LAYEK et al. 2015). For instance, the mechanism of uptake of arginine rich CPPs such as polyarginine or TAT into the cell has been demonstrated to occur by macropinocytosis where cell membrane ruffling plays a crucial role (KRISTENSEN et al. 2016). Cellular uptake of unconjugated TAT peptide is related to clathrin-mediated endocytosis, whereas the uptake of conjugated TAT occurs by caveolae-mediated pinocytosis (RICHARD et al. 2005).

Natural α -peptides are rapidly degraded by proteases (HOOK et al. 2005). This is a major concern regarding applying these molecules as CPPs for drug delivery (BRUNO et al. 2013). To overcome proteolytic degradation, change of conformation (such as using the D-isomer) or β -peptides are suggested to increase stability, the latter being synthetic and consisting of homologated proteinogenic amino acids, and are considered to be better suited as CPPs (KAMENA et al. 2011).

By conventional solid-phase peptide coupling, β 3 oligoarginine (β -OA) was synthesized from the monomeric building block Fmoc- β 3hArg(Boc)₂-OH. β -OA has been used in biological investigations with promising results. It has been proven to penetrate into bacterial cells, like *Escherichia coli* and *Bacillus megaterium*, into mouse fibroblast, HeLa cells and Human Foreskin Keratinocytes (HFK). The success of penetration into HeLa cells depends on the chain length of transported peptides. Short chain peptides (tetramer and hexamer) were found to stick on the cell surfaces while long chain peptides (octamer and decamer) accumulated inside the cytoplasm following penetration into the cells and ended up in the nuclei. Penetration of β -OA into HFK cells worked both at 4 °C and 37 °C and in the presence or absence of cellular metabolism disruptor (NaN₃) (SEEBACH et al. 2004). Moreover, β -OA was tested for induction of hemolysis in rat and human erythrocytes and was found to be non-hemolytic even at a concentration as high as 100 μ M.

In a previous study, fluorescent-labeled β -OA was used to track infected erythrocytes. It was found that β -OA can enter different cell types such as macrophages, fibroblasts, and hepatocytes by various

pathways. Interestingly, β -OA can only go through membranes of erythrocytes when they are infected by *Plasmodium* (KAMENA et al. 2011) and failed to overcome the intact membrane of healthy human erythrocytes. From this intriguing result it appears that β -OA may be an interesting candidate for delivery of antimalarial drugs to the site of parasite infestation. Octaarginine was applied to increase the efficacy of fosmidomycin against *Plasmodium* and *Mycobacterium in vitro* (SPARR et al. 2013). Coupling with octaarginine increased the efficacy of fosmidomycine in malaria treatment dramatically ($IC_{50} = 4.4$ nM) compared with fosmidomycine alone ($IC_{50} = 181.4$ nM). *In vivo* it was shown that β -OA can even penetrate tissue layers and may thus serve as a transporter e.g. through the skin as demonstrated *in vivo* in a laboratory mouse model (SEEBACH et al. 2004).

2.9 Electroporation and electroporation-free transfection

Electroporation is an efficient method using high voltage electric pulses to introduce foreign genes into cells to produce transient transfection. However, the transfection by electroporation requires large amounts of DNA plasmid of up to 100 μ g (POTTER and HELLER 2003). Besides, high voltage application may not be suitable for transfection of protozoan parasites and may be harmful e.g. apicomplexan sporozoites. Therefore, the amount of *C. parvum* sporozoites used for electroporation has to be quite high with up to 10^7 (VINAYAK et al. 2015, PAWLOWIC et al. 2017). Other options of transfection have been studied to overcome problems associated with electroporation. For example, cationic polyethylenimine (PEI) was successfully applied to deliver green fluorescent GFP gene into the genome of the protozoan parasite *Toxoplasma gondii* (SALEHI and PENG 2012). In fact, due to the electrostatic interaction between the cationic polymer and the negatively charged DNA plasmid, the resulting complex protected the DNA from degradation and facilitated uptake of the complex into the host cells (SMEDT et al. 2000). Therefore, PEI can be considered for transfection applications for other protozoan parasites such as *C. parvum*, however, no respective data have been published so far.

3. Results

3.1 Publication 1: A simple and efficient transfection protocol for *Cryptosporidium parvum* using Polyethylenimine (PEI) and Octaarginine

Tran Nguyen-Ho-Bao, Maxi Berberich, Wanpeng Zheng, Dieter Seebach, Arwid Dauschies, Faustin Kamena.

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Author's contribution

1. Concept

Tran Nguyen-Ho-Bao was responsible for the idea and design of experiments under the supervision by Faustin Kamena and Arwid Dauschies.

2. Investigation

Tran Nguyen-Ho-Bao was responsible for conducting the experiments and collecting data with the support of Wanpeng Zheng (transfection protocol), Maxi Berberich (optimization excystation) and Dieter Seebach (synthesis of compound).

3. Analysis

Tran Nguyen-Ho-Bao performed the data analysis, interpreted the results with the help of Faustin Kamena and Arwid Dauschies.

4. Manuscript

Tran Nguyen-Ho-Bao was responsible for writing with the support by Maxi Berberich and Wanpeng Zheng, Faustin Kamena and Arwid Dauschies.

3.2. Manuscript 2: Octaarginine significantly improves the efficacy of nitazoxanide against *Cryptosporidium parvum*

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Authors contribution

1. Concept

Tran Nguyen-Ho-Bao was responsible for the idea and design of experiments under the supervision by Faustin Kamena.

2. Investigation

Tran Nguyen-Ho-Bao was responsible for conducting all *in vitro* and *in vivo* experiments with the support of Maxi Berberich (*in vivo* experiments). Histopathology was performed by Christiane Helm.

3. Analysis

Tran Nguyen-Ho-Bao performed data analysis and interpretation of results with support by Faustin Kamena and Arwid Dauschies. Histopathology data were analysed by Christiane Helm and Reiner Ulrich.

4. Manuscript

Tran Nguyen-Ho-Bao was responsible for writing with support by from Maxi Berberich and Christiane Helm. Thomas Grunwald, Reiner Ulrich, Faustin Kamena and Arwid Dauschies critically revised the manuscript and proposed improvements in styles and contents.

Octaarginine significantly improves the efficacy of nitazoxanide against *Cryptosporidium parvum*

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Abstract

Cryptosporidiosis is an intestinal disease which occurs in a variety of hosts including animals and humans. Until now, there is no vaccine available for this disease. Nitazoxanide (NTZ) is the only FDA approved drug for cryptosporidiosis treatment in man, however its efficacy in immunocompromised people such as AIDS patients or malnourished children is limited and it is not licensed for animals. A major obstacle faced by drugs against intracellular pathogens is the transport of the drug into the infected cell and into the parasitophorous vacuole. In this study, we have investigated the potential of the cell-penetrating peptide octaarginine to increase the uptake of NTZ by cells and thereby to improve its efficacy. For this purpose, octaarginine was synthetically attached to NTZ and the resulting complex NTZ-R8 tested for the inhibition of *Cryptosporidium* growth in comparison to unmodified NTZ. The inhibitory properties of NTZ and NTZ-R8 were tested both *in vitro* using the human ileocecal adenocarcinoma (HCT-8) cell line as well as *in vivo* on the IFN- γ -knockout mouse model. Both *in vitro* and *in vivo* we observed a distinct improvement of drug efficacy when NTZ was attached to octaarginine. Particularly, the improved survival of infected mice treated with NTZ-R8 encourages further evaluation of application of octaarginine as a vehicle for anticryptosporidial compounds to develop alternative treatment solutions against this infection.

Key words: *Cryptosporidium*, Nitazoxanide, Octaarginine, IFN- γ knockout mice

Author summary

Cryptosporidiosis is an important and widely distributed intestinal disease induced by the apicomplexan parasite *Cryptosporidium parvum* in neonate animals and in humans. It can cause severe diarrhoea leading to high mortality in young ruminants, infants and immunodeficient patients. Currently, no vaccine is available to prevent cryptosporidiosis. Moreover, for man only nitazoxanide (NTZ) is approved in the USA. However, NTZ proved to be of limited

efficacy in immunocompromised persons and is not licensed for using in veterinary medicine. Developing novel anti-cryptosporidal compounds or increasing the efficacy of existing drugs is an urgent need to achieve satisfactory treatment options for *Cryptosporidium* infection in both man and animal. In this study, we evaluated the property of the cell-penetrating peptide (CPP) octaarginine to improve the efficiency of NTZ on *Cryptosporidium parvum* by coupling octaarginine to nitazoxanide (NTZ-R8). NTZ-R8 showed significantly higher efficacy as compared to NTZ alone in terms of growth inhibition *in vitro* and increased survival of IFN- γ -knockout mice.

Introduction

Cryptosporidium spp. is a small protozoan parasite which causes gastroenteritis in both animals and humans worldwide. Due to the wide range of hosts and various transmission pathways, *Cryptosporidium parvum* has not only a tremendous impact on animal health but also needs to be considered in the context of the “One Health” concept. Cryptosporidiosis in humans is characterized by diarrhea, nausea, vomiting, fever, and abdominal pain; however, the disease is self-limiting in persons with an intact immune system [1,2]. In contrast, infection of immunocompromised patients or malnourished children with either *C. parvum* or *C. hominis* may become life-threatening without proper treatments [3,4]. The introduction of highly active antiretroviral therapy (HAART) against HIV infection has significantly reduced the incidence of fatal *Cryptosporidium* infection in industrialized countries [5,6]. Nevertheless, *Cryptosporidium* infection was listed in 2013 by the Global Enteric Multicenter Study (GEMS) as the second leading cause of diarrhoea-associated mortality in children in developing countries [4]. While *C. parvum* is zoonotic and can cause severe disease in both man and animals, particularly young ruminants, *C. hominis* infects exclusively humans. There are no vaccines available against cryptosporidiosis and therapeutic options to treat and control the

disease are rather limited. For chemotherapy of human cryptosporidiosis, nitazoxanide (NTZ) is the only FDA-approved drug. However, NTZ shows poor efficacy in immunocompromised patients and malnourished children [7, 8] and is not licensed for use in animals. The development of new or alternative therapeutic strategies to control cryptosporidiosis is a necessity. However, developing fully new drugs is a time consuming and costly process. Alternatively, repurposing drugs that are established for other indications or increasing the efficacy of available anti-cryptosporidial compounds represent attractive options for widening the repertoire of therapeutic solutions. In this study, we opted for the use of the short cell-penetrating peptide octaarginine to increase uptake of NTZ into *Cryptosporidium*-infected cells and thereby improve its efficacy.

In recent years, the short polycationic peptide octaarginine has been successfully used as a delivery vehicle for drugs as well as plasmid DNA into intracellular parasites [9,10,11]. It has been demonstrated that octaarginine dramatically improves efficacy of the experimental anti-malaria drug fosmidomycin [10]. In the present study, we explored whether coupling of octaarginine to the anti-*Cryptosporidium* drug NTZ facilitates its uptake by infected cells thus possibly improving anticryptosporidial efficacy [9 ,10]. For this purpose, octaarginine was coupled to NTZ to create the NTZ-R8 conjugate. Importantly, the octaarginine moiety was coupled to NTZ via an ester bond and can be released by esterase. The conjugated compound was tested both *in vitro* and *in vivo*. We found in both cases a distinct improvement of anti-cryptosporidial activity compared to NTZ alone. These results encourage further investigations to clarify the usefulness of this strategy for development of an alternative therapeutic approach against cryptosporidiosis.

Material and methods

Compounds

Results

Nitazoxanide (NTZ) (Sigma-Aldrich, Darmstadt, Germany) and nitazoxanide-octaarginine (NTZ-R8) were dissolved in DMSO (10 mg/ml) and stored in the dark at -20°C. The drugs were freshly prepared in infection medium (DMEM supplemented with 2% fetal calf serum, 1% antibiotics penicillin/streptomycin, and 1% amphotericin B) for *in vitro* and *in vivo* testing.

Cell culture

In the *in vitro* study, human ileocecal adenocarcinoma cells (HCT-8) were seeded into 24-well plates at an initial density of 2×10^5 cells/well. The cells were grown up to 70-80% cell confluence in 1-2 days in RPMI medium. The growth medium consisted of RPMI medium supplemented with 10% fetal calf serum (FCS) (Northumbria, Cramlington, UK), 1% antibiotics (penicillin/streptomycin), and 1% amphotericin B.

Parasites

Cryptosporidium parvum strain (gp60/ subtype IIa A15G2R1) was isolated from calves (Köllitsch, Germany). *C. parvum* oocysts were passaged every 3 months in calves under experimental conditions, and the oocysts were purified from feces following the protocol described by Najdrowki et al [12]. Oocysts were stored in PBS, pH= 7.2 (Gibco®, ThermoFisher Scientific, Massachusetts, USA) supplemented with penicillin/streptomycin (200 µg/ml) and amphotericin B (5 µg/ml) to prevent bacterial and fungal growth at 4°C for up to 3 months until use. The storage medium was replaced every 2 weeks. Before usage, the oocysts were bleached with cold NaOCl (5.25% diluted 1:1 (v/v) in PBS; pH = 7.2) by incubating on ice for 5 min. Oocysts were then washed extensively with cold PBS (3 times) to completely remove NaOCl before excystation. In order to obtain free sporozoites, oocysts were resuspended in excystation medium (sodium taurocholate -NaT at a final concentration of 0.4% in DMEM medium supplemented with 2% fetal calf serum, 1% penicillin/ streptomycin, 1% amphotericin B, and

1% of sodium pyruvate) and processed following the protocol as described in Nguyen-Ho-Bao et al [11].

Uptake of FAM-labeled octaarginine by excysted sporozoites and intracellular *Cryptosporidium parvum*

6-FAM-labeled octaarginine (10 µg/ml) was added to either free sporozoites or HCT-8 cell cultures infected by intracellular stages of *C. parvum*. Intracellular 6-FAM-labeled octaarginine was visualized by direct fluorescence and/ or immunofluorescence assay was applied to detect *C. parvum* using a Leica TCS SP8 laser scanning confocal microscope (Leica, Wetzlar, Germany). Sporozoites were centrifuged at 9500 x g for 5 min, followed by a washing step with PBS (pH =7.2). All steps of the following protocol were performed at room temperature. Sporozoites or infected host cells were fixed with 4% paraformaldehyde (PFA) for 20 min, and thereafter washed 3 times with PBS. Then, 4,6-diamidino-2-phenylindole (DAPI; 10µg/ml) was added followed by incubation for another 5 min. In the immunofluorescence assay, the permeabilization with 0.2% Triton X-100 for 20 min was performed right after the fixation step. Then, 1 % bovine serum albumin (BSA) in PBS was added to block unspecific binding. Thereafter, infected cells were incubated for 1 h with a specific primary rat-anti *Cryptosporidium* antibody (Waterborne INC, New Orleans, LA, USA) in PBS containing 1% BSA at a dilution of 1: 1000. Goat-anti-rat Dylight™ 647 (Rockland, Limerick, USA) was used as secondary antibody and the nuclei stained with DAPI. Finally, cells were mounted with Fluoromount-G (Southern Biotech, Brimingham, USA) and stored at 4°C until visualization.

Mitochondrial toxicity test (MTT)

For MTT 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide was applied to evaluate whether NTZ and octaarginine at the chosen concentrations have a negative impact on host cell viability. A total of 7 x 10⁴ HCT-8 cells were seeded into 96 well-plates and incubated at 37°C

Results

under 5% CO₂ until the cell cultures reached 80% confluence. NTZ (25, 20, 15, 10, 5, 1 µg/ml) and octaarginine (100, 10 and 1 µg/ml) were freshly diluted in culture medium and added to the growing cultures for 24 h. Subsequently, 10 µl MTT solution (containing tetrazolium dye) were added to each well and the plates were further incubated for 4 h. Stop solution (10% SDS/ 0.01 M HCl) was added to dissolve precipitates of formazan crystals. Absorption was measured by spectrophotometry (Anthos 2001) at 595 nm. Each concentration was set in triplicates.

***In vitro* inhibition assay**

HCT-8 cells were seeded into 24-well plates (2 x 10⁵ cells/well) and incubated until they reached a confluence of 80%. Cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, antibiotics (1% penicillin/streptomycin, 1% amphotericin B), and 1% sodium pyruvate. They were incubated at 37°C with 5% CO₂. Confluent monolayers were inoculated with 2 x 10⁵ oocysts in excystation medium (0.4% NaT in DMEM with 2 % fetal calf serum, 1% amphotericin B and 1% penicillin/streptomycin, 1% sodium pyruvate) and further incubated at 37°C and 5% CO₂ for 3 h. Non-excysted oocysts and empty oocyst shells were gently removed by washing with PBS for 3 times. NTZ and NTZ-R8 were diluted in growing medium at different concentrations (1, 5, 10, 50, 100, 1000 ng/ml) and added to cell cultures previously exposed to infection and further incubated (37°C, 5% CO₂) for 24 h. Uninfected untreated cells served as negative controls. Positive controls were infected cultures that were not treated with NTZ or NTZ-R8, respectively. All conditions were set as triplicates.

RNA extraction. Exactly 24 h post infection, cell culture plates were centrifuged at 1000 x g for 10 min to ensure that remaining extracellular stages of the parasite firmly settle on the bottom of the well. The culture medium was gently aspirated and cells were harvested by directly adding lysis buffer of the RNeasy® Mini Kit (Qiagen, Hilden, Germany) to each well. Further extraction of RNA from the samples was done strictly following the instructions of the manufacturer. Total RNA was measured by a NanoPhotometer NP80 (Implen, Munich,

Results

Germany). 1 µg RNA was used to produce respective cDNA according to the instruction delivered with the Revert-Aid® first strand cDNA synthesis kit (Thermo Fisher Scientific, Darmstadt, Germany). The cDNA was stored at -80°C until further use.

Real-time PCR. Real-time PCR reactions were performed on a Bio-Rad CFX96 Real-Time PCR Detection System using the program two-step SYBR green with primers for *Cryptosporidium* 18S RNA targeting Cp18S-1011F (5'-TTG TTC CTT ACT CCT TCA GCA C-3') and Cp18S-1185R (5'- TCC TTC CTA TGT CTG GAC CTG-3'). The data were normalized by the transcription levels of host cell Hs18S rRNA [13] , applying the primer pair Hs18S- 1F (5'-GGC GCC CCC TCG ATG CTC TTA-3') and Hs18S- 1R (5'-CCC CCG GCC GTC CCT CTT A-3'). The thermo cycler program for RT-qPCR was: 95°C for 3 min, followed by 40 amplification cycles at 95°C for 10 s and 58°C for 30 s. The melting curve analysis was performed at a temperature range between 65°C and 95°C. The transcription level of Hs18 rRNA was applied for both normalization and controls. The following formula were used to estimate parasite growth inhibition (PGI%)

$$\Delta C_T = C_{T[Cp18S]} - C_{T[Hs18S]}$$

$$\Delta\Delta C_T = \Delta C_{T[sample]} - \Delta C_{T[control]}$$

$$PGI \% = 100 - (2^{-\Delta\Delta C_T}) \times 100$$

Titration of infection dose in IFN-γ knockout mice

IFN-γ knockout female mice aged 6-12 weeks were randomly pooled into 4 groups of 3 mice each. Group 1 served as uninfected control group, whereas mice in groups 2, 3, and 4 were inoculated with *C. parvum* oocysts at a dose of 10000, 5000, or 1000 oocysts per mouse, respectively. Determination of individual weight, collection of feces, and scoring of general health were performed daily until 13 days post infection.

***In vivo* assessment of drug efficacy in INF- γ knockout mice**

Animal experiments were approved by the local ethical committee and the local authority (Landersdirektion Sachsen, Germany) under the number TVV 07/19. IFN- γ -knockout mice were randomly pooled into 3 groups (n = 5). All mice were inoculated by oral gavage with 1000 oocysts. The trial design is shown in Figure 1. The infected mice were treated daily from day 6 post infection (dpi), either with 5% DMSO in water (sham treatment), 10 mg NTZ/kg BW group, or 2 mg NTZ-R8/kg BW group. All mice were weighed and scored daily by two different persons to avoid individual bias. Fecal samples were collected daily.

Figure 1: Experimental design of drug evaluation in the mouse experiment.

Estimation of oocyst excretion

Feces were collected and pooled for each cage daily. Feces were stored at -20 °C until DNA extraction. To calculate the number of oocysts passed in 1 g of feces (oocysts per gram = OpG), DNA was extracted from 100 mg mouse feces using the Fast DNA Stool kit (Qiagen, Germany) according to the instructions of the manufacturer. Quantitative (q)PCR was performed following the protocol described in Shahiduzzaman et al [14]. PCR primers amplifying the *C. parvum* hsp70 gene (forward primer 5'-AACTTTAGCTCCAGTTGAGAAAGTACTC -3'; reverse primer: CP_hsp70_rvs 5'-CATGGCTCTTTACCGTTAAAGAATTCC 3'; TaqMan

Results

probe: HSP_70_SNA 5'-AATACGTGTAGAACCACCAACCAATACAACATC-3') were used along with the following cycler program: denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 s, and annealing at 60°C for 1 min. The total volume of each PCR reaction contained 25 µl consisting of 1X Master Mix (Thermo Fisher Scientific, Darmstadt, Germany) 0.3 µM of forward primer and 0.9 µM of reverse primer, 0.2 µM of Hsp70 probe labelled at the 5'-end with the 6-carboxyfluorescein reporter dye (FAM) and at the 3'-end with the 6-carboxytetramethylrhodamine quencher dye (TAMRA). The qPCR reactions were performed applying the Bio-Rad CFX96 Touch Real-Time PCR Detection System and PCR plates (Bio- Rad Laboratories, Hercules, CA). For qPCR, purified plasmid was used to construct the standard curve. OpG were calculated based on the DNA copies as followed: (with n= number of DNA copies, a: dilution DNA in µl water, b: milligram of feces and 4 = number of sporozoites in one oocyst)

$$\text{OpG} = n \times \frac{a}{5} \div 4 \times \frac{1000}{b}$$

A sample containing feces obtained from non-infected mice but spiked with known number of freshly isolated oocysts was also processed in order to evaluate the correlation of qPCR and the actual number of oocysts.

Histopathology

Mice were euthanized at day 14 post infection or died during experiment (dead in cage or euthanized when they lost more than 20 % of their body weight). For each mouse, a specimen from the ileum was collected 2 cm anterior to the cecum. The fragments were flushed and fixed in 4% formaldehyde. Fixed samples were processed, embedded in paraffin and were cut into slices of 2-3 µm thickness slices were deparaffinized and stained with hematoxylin and eosin. Histopathologic evaluation and documentation was performed using an Olympus BX53

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microscope with 2x, 4x, 10x, 20x, 40x, and 100x-oil-immersion objectives and 5 megapixel digital colour camera (Olympus Deutschland GmbH, Hamburg). The average villus to crypt ratio was estimated and the density of *C. parvum* organisms present in the mucosal epithelium. The severity of inflammatory infiltrates in the lamina propria and submucosa were graded on a scale from 0 = not present, 1 = oligofocal organisms; mild inflammation, 2 = multifocal organisms; moderate inflammation, 2 = disseminated organisms; severe inflammation.

Data analysis

Data obtained by qPCR were analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA, US). Statistical analyses and graphs were done using GraphPad Prism version 8.02 (GraphPad Software, Inc., La Jolla, California, US). The survival rate was evaluated by Kaplan Meier plot and p-values for survival were calculated by Mantel Cox test. Data used for IC₅₀ calculation for NTZ and NTZ-R8, and for estimation of the dose-response-inhibition were performed to previous logarithmic transformation.

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Uptake of FAM-labelled octaarginine by *Cryptosporidium*

6-FAM-labeled octaarginine was supplemented to either freshly excysted *C. parvum* sporozoites or *C. parvum*- infected HCT-8 cell cultures. For extracellular sporozoites, the peptide uptake occurred rather rapidly, and within 10 min octaarginine accumulated in the parasite nucleus (Figure 2A). The incubation of FAM-labelled octaarginine with *C. parvum*-infected HCT-8 monolayers revealed that octaarginine entered the parasitophorous vacuole within 1 h of incubation (Figure 2B). These observations confirm the potential of octaarginine to pass across membranes into both invasive and intracellular stages of *C. parvum*.

Figure 2: (A) Freshly excysted *C. parvum* sporozoites were incubated with 6-FAM-Octaarginine (green) for 30 min prior to analysis. (B) Logarithmic culture of *C. parvum* in HCT-8 cells were incubated with FAM-octaarginine for 30 min prior to analysis. Multiple stages of the parasites can be seen bearing the green color of the labelled peptide. DAPI was used to visualize the nuclei and anti-Crypto antibodies (Red)

NTZ-R8 inhibits *C. parvum* growth in HCT-8 cells

Nitazoxanide was coupled synthetically to octaarginine in order to generate the conjugate molecule, Nitazoxanide-octaarginine (NTZ-R8). An ester bond was used for the coupling of two moieties and shall enable the release of the tizoxanide, the active molecule, upon hydrolysis by esterase (Figure 3)

Figure 3: The chemical structures of tizoxanide, nitazoxanide (NTZ) and nitazoxanide-octaarginine (NTZ-R8). Red arrow showing the cleavage position by esterase to release tizoxanide from NTZ-R8

Before evaluating the activity of NTZ-R8 on the target organism, *C. parvum*, potential cytotoxic effects of NTZ and octaarginine on host cells HCT-8, were evaluated using the MTT test. Concentration ranges of 1 to 25 μg NTZ/ml and of 1 to 100 μg octaarginine /ml were tested. For both substances no relevant cytotoxicity was found, although cell viability appeared to be slightly reduced at the very high and non-physiological doses applied (Figure 4)

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Figure 4: Viability of HCT-8 cells following exposure to NTZ (A) and octaarginine (B) over a period of 24 h of drug exposure.

In order to test the inhibitory properties of the drugs, HCT-8 cells were infected with *C. parvum* and treated with NTZ or NTZ-R8 at 6 different concentrations (1, 5, 10, 50, 100, 1000 ng/ml). These treatment doses have been previously tested by MTT and no toxicity was observed. At the highest concentration of 1000 ng/ml, NTZ-R8 and NTZ distinctly inhibited *C. parvum* growth by 97.57% and 79.98%, respectively (Figure 5). IC_{50} was 60.54 ng/ml (197 nM) for NTZ and 4.499 ng/ml (2.9 nM) for NTZ-R8 (Figure 5). Coupling of NTZ to octaarginine (NTZ-R8) thus significantly increased the inhibitory efficacy of NTZ on *C. parvum* growth *in vitro* with $P=0.0045$.

Figure 5: Growth inhibition of *C. parvum* in HCT-8 monolayers by NTZ and NTZ-R8. IC_{50} NTZ= 60.54 ng/ml (197 nM) and IC_{50} NTZ-R8= 4.499 ng/ml (2.9 nM). The data presents $\pm$ SEM. The value of IC_{50} is calculated by Graphpad 8.1.

Titration of infection dose in IFN- γ knockout mice

Following successful evaluation of the effect of octaarginine on NTZ efficacy *in vitro*, we decided to continue the evaluation *in vivo* using the established IFN- γ knockout mouse model of cryptosporidiosis. Because reports concerning the required number of *C. parvum* oocysts to mount an infection in the mouse are not unanimous, we decided to assess the optimal number of oocysts for our experiments. For this purpose, we conducted titration applying different numbers of oocysts to infect the mice as followed: G1 (no infection), G2 (10 000 oocysts) G3 (5000 oocysts) and G4 (1000 oocysts). As shown in figure 6, OpG increased in all infected groups (G2, G3, and G4). Although there were no significant differences in oocyst shedding between different groups irrespective of the infection dose, mice with the highest inoculation dose of 10000 oocyst all mice died on the day 8 while mice of group 3 (5000 oocysts) and group 4 (1000 oocysts) survived until 11 dpi and 13 dpi, respectively. This indicates that the oocyst shedding is not necessarily a read out for disease pathology. For further experiments we decided to use the lowest infection dose of 1000 oocysts. All infected mice in the 3 groups revealed the

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clinical symptoms for cryptosporidiosis such as emaciation, dehydration reducing food consumption, trembling, ruffled fur and reluctance to move.

Figure 6: Oocysts shedding of IFN- γ knockout mice measured by qPCR. G1: non-infected group, G2, G3 and G4: mice were challenged with 10000, 5000 and 1000 oocysts, respectively (n= 3).

Coupling of NTZ to octaarginine (NTZ-R8) significantly improves the efficacy on cryptosporidiosis in the IFN- γ knockout mouse model.

In order to assess the effect of NTZ-R8 in the mouse model, several clinical parameters were recorded. The weight of mice belonging to the untreated infected control group showed a sharp decrease from 6 until 11 day post infection (dpi). Weight loss was also recorded in the two treated groups; however, it occurred less rapidly. At 8 dpi, the mice in the control group huddled together in a corner of the cage, fur became ruffled, the animals appeared emaciated (weight lost nearly 15%) and feces was soft. On 9 dpi two mice died and one mouse was euthanized to prevent further suffering since the humane endpoints has been reached. Mice were reluctant to move, were hunched and displayed a late response to stimulation. In contrast to the sham treated group, mice treated either with NTZ or NTZ-R8 experienced a less dramatic weight loss which result in a better survival. Strikingly, weight loss mice treated with NTZ-R8 was stabilized from

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10 dpi and even increased slightly in the last two days of the experiment for most of the animals (Figure 7). In addition to stabilizing their weight loss, treated animals also showed a significant improvement in their active movements and on immediate response under stimulation. Only one mouse of the NTZ treated group showed a late response to stimulation at day 12

Figure 7: The percentage of weight loss of every IFN- γ knockout mouse during infection by *C. parvum*. Treatment with NTZ or NTZ-R8 was given during the period 6 dpi to 13 dpi once per day.

Survival rate

When comparing the survival rate, we noticed that it dropped dramatically in the sham treated group to 40% within 9 dpi and reached 0% by 11 dpi. For the mice group treated with NTZ alone (10 mg/kg BW), survival rate dropped to 60% on day 11 and reached 40% by the end of experiment at 13 dpi. Strikingly, mice treated with the NTZ-R8 conjugate (2 mg/kg BW) showed a better survival with only one mouse dying at 10 dpi and all remaining animals surviving until the end of the experiment (Figure 8). Despite the obviously higher survival of NTZ-R8 treated animals compared to NTZ only treated animals, the difference was not statistically different ($P > 0.05$). Due to limited availability of the synthetic conjugate NTZ-R8,

the highest concentration used was 2 mg/kg BW whereas a 5-fold higher dose (10 mg/kg BW) was used for NTZ alone.

Figure 8: Kaplan –Meiser curve for survival estimation of *C. parvum* infected INF- γ knockout mouse. Mice were infected with 1000 oocyst in 0 dpi and gave treatment at 6 dpi. Sham treated group served as positive control with DMSO 5%, NTZ: treated group with nitazoxanide at the concentration 10 mg/kg BW, and NTZ-R8: treated group with nitazoxanide-octaarginine at the concentration 2 mg/kg BW

Oocyst shedding

All infected mice started shedding oocysts at 2 dpi. Feces samples were pooled per group and analyzed by qPCR. On 7 dpi, the mice of shame treated group (5% DMSO/ water) excreted 3.83×10^8 oocysts/g feces. In the mice treated with NTZ (10 mg/kg BW) or NTZ-R8 (2 mg/kg BW), oocyst shedding was reduced as early as the 7 dpi (24 h after initiation of treatment) with only 1.19×10^7 and 2.12×10^7 oocysts/g feces, representing a reduction of oocyst excretion of 96.91 % and 94.49 %; respectively (Figure 9). Oocyst reduction in both treated groups was significant at 7 dpi ($P < 0.001$). These *in vivo* results are consistent with the inhibition of reproduction documented in the *in vitro* testing in HCT-8 cells.

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During the following 4 days of treatment (6 dpi to 9 dpi), oocyst shedding was still reduced but on a lower level than initially observed (67.59 % and 62.34 % for NTZ and NTZ-R8, respectively). Further comparison of groups was not possible because all sham treated mice died by 11 dpi.

Figure 9: Average OpG (logarithmic scale) estimated by qPCR over 13 days of observation. Mice were infected with 1000 oocysts on study day 0 and treated at 6 dpi. Data shown are mean $\pm$ SEM (n=3)

Histopathology analysis

The non-infected mouse showed a villus to crypt ratio of 3:1 and no inflammatory infiltrates in the ileal mucosa (Figure 10: A-C). In contrast, all infected mice displayed a qualitatively comparable, moderate to severe, subacute, diffuse, proliferative and variably lymphohistioplasmacytic ileitis (Figure 10 D-L). The obvious combination of crypt hyperplasia and villus atrophy induced a marked drop in the villus to crypt ratio to a median of ≤ 1 in all infected mice (Figure 10 D-L). Detached and degenerating epithelial cells, occasionally forming crypt abscesses were present within the crypt lumina. There was a trend to more severe

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inflammatory infiltrates in the mucosa in the NTZ-R8 treated mice as compared to the NTZ and the sham treated mice. Large numbers of *C. parvum* stages were located at the apical epithelial surface. In mice treated with NTZ or NTZ-R8, a trend towards a reduced density of parasites was seen as compared to the sham treated group

Figure 10: Histopathological analysis of infected animals after treatment.

The ileum of non-infected mice (A-C) is shown in comparison to that of *Cryptosporidium parvum*-infected mice treated with either DMSO (D-F), NTZ (G-I) or NTZ-R8 (J-L). Arrowhead in E showed the degenerating and detached epithelia within the crypt lumina. Arrows represented *C. parvum* at the apical surface epithelium. Bars A, D, G, J = 100 µm; B, E, H, K = 50 µm; C, F, I, L = 20 µm.

Discussion

Nitazoxanide (NTZ) is the only FDA approved drug for cryptosporidiosis treatment in human. However, controversial results regarding efficacy of NTZ in cryptosporidiosis treatment in children and HIV patients exist. In particular, NTZ showed limited efficacy in cryptosporidiosis treatment in HIV patients in different studies [7, 15 ,16] and in immunocompromised mice [17]. In general, the major obstacle comes from the lack of sufficient efficacy of available drugs licensed for treatment of *Cryptosporidium* infection. As an alternative to efforts aiming at developing new drugs, we focused on empowering an existing drug as to render it more efficacious. For this purpose, NTZ, an established anti-cryptosporidial drug was coupled to the short polycationic cell-penetrating peptide octaarginine to anti-parasitic drugs such as the anti-malarial experimental drug fosmidomycin dramatically improved drug efficacy to staggering 40% [10].

NTZ is a pro- drug that is rapidly hydrolyzed by esterase and transformed into its desacetyl derivative, tizoxanide, the active metabolite [18]. Considering this feature, octaarginine was coupled to NTZ in order to release tizoxanide upon esterase cleavage (Figure 3). Octaarginine has been demonstrated to cross various biological membranes such as the blood brain barrier [19] and membranes of different cell types [9, 20]. Therefore, to evaluate a potential use of octaarginine as delivery vehicle for NTZ in *Cryptosporidium* treatment, we first assessed the permeation of octaarginine across parasites membrane as well as cell membranes. The limiting factor for a CPP such as octaarginine to function as carrier for any cargos into a cell is the permeability across the corresponding biological membrane and it is totally independent of the size of cargo [9, 21]. FAM-labelled octaarginine was used to track the permeation of this CPP through the parasite and host membranes. We found that octaarginine was taken up more rapidly by free sporozoites of *Cryptosporidium* than by intracellular stages. This can be

explained by the special location of intracellular *Cryptosporidium* which is extracytoplasmic [22]. In fact, although *Cryptosporidium* species are obligate intracellular parasites, they never enter host cytosol but remain throughout their entire intracellular life in an extracytoplasmic space directly underneath the host plasma membrane [23]. The parasites are thus protected by the host generated membranes of the parasitophorous vacuoles [24] that is a barrier for NTZ to cross thus preventing drug accumulation in the parasite sufficient to achieve optimal activity on the target. Our findings show that octaarginine penetrates all membranes surrounding the parasite in a fast kinetic than the host cell membrane. A strong staining was observed in the parasite after less than 30 min incubation while the host cell remained unstained. This results suggested that coupling of NTZ to octaarginine represent an attractive alternative to increase NTZ accumulation in the parasite.

Concording with the above-mentioned results on the permeability of the parasite membrane to octaarginine, we observed during the *in vitro* experiments that NTZ coupled with octaarginine (NTZ-R8) inhibits the growth of *C. parvum* in HCT-8 cells more efficiently than NTZ alone after 24 h of exposure. The calculated IC₅₀ of NTZ- R8 was 2.9 nM while that of NTZ alone was 197 nM. By facilitating the transport of NTZ across the host cell membrane as well as through the membrane of the parasite, octaarginine improved the activity of NTZ more than 60-fold. In our study, NTZ at a dose of 1000 ng/ml inhibits *C. parvum* replication to 79% that is higher than published before [23, 24, 25]. Although NTZ can be dissolved in DMSO, we recognized that precipitation occurred after further dilution in DMEM culture medium. Therefore, we applied sonication for 30 s (2-3 times) to ensure complete dissolution in the medium before adding to infected cells. It may be assumed that this additional procedure increased the activity of NTZ to a level higher than in former studies.

Although many drugs have been shown to successfully inhibit *Cryptosporidium in vitro*, e.g. monensin, halofuginone, paromomycin [14, 25], the inhibition in animal models was less

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obvious [16]. There are many elements such as bioavailability, pharmacokinetics, food-drug interaction [28] which influence the efficacy in animals and that partly explain the difficult transferability of *in vitro* results to *in vivo* conditions [29].

In order to further investigate the effect of NTZ-R8, we decided to assess the conjugate molecule in an animal model. For this purpose, IFN- γ knockout mice were used because they are generally considered highly susceptible to *C. parvum* [17, 29, 30] and suitably mimic the response of immunocompromised patients to this parasite. Other mouse models such as SCID [31], neonate mice with infection doses from 1×10^3 to 2.5×10^4 oocysts [32,33,34], or dexamethasone-treated mice [35] require high infection dose (i.e 10^6 oocysts), while IFN- γ -knockout mice may display severe illness after inoculation of only 10 oocysts [30]. IFN- γ -knockout mice have been used to analyze the impact of drug therapy on acute infection [36]. The titration results showed that regardless of how many oocysts (1000, 5000 or even 10000 oocysts) were used for infection, the peak of infection was recorded at 8 dpi. The severity of sickness was independent on the number of inoculated oocysts. However, the main difference among 3 groups was the prepatency. Infection with the highest dose 10000 oocysts resulted in patency at 1 dpi while the earliest excretion was observed on 2 dpi in infected mice with lower infection dose of 5000 or 1000 oocysts. Therefore, to reduce unnecessary suffer for mice, lowest tested dose of 1000 oocysts was applied in further *in vivo* experiments. This inoculation dose was lethal (reaching humane endpoints or animals dead) between 8 to 14 dpi [36]. Basing on the development of clinical symptoms of infected mouse with *Cryptosporidium* in our trial experiment as well as previous study described by Manjunatha [17], the treatment was applied on 6 dpi to evaluate the drug efficacy. In general, the typical clinical symptoms of cryptosporidiosis such as emaciation, anorexia, dehydration, ruffled fur, and a high level of oocyst excretion were recorded as previously described in mouse models. The most profound symptom of cryptosporidiosis in neonate calves [36, 37, 38] and humans [4, 39] is diarrhea,

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however the development of diarrhea was not obvious in the current mouse experiment which is a general limitation of mouse models [40]. Consequently, it was not possible to evaluate the efficacy of the applied treatment on this clinical parameter. This confirms that results obtained in laboratory rodent models in terms of cryptosporidiosis control have to be interpreted with caution. Since the increased survival in the treated groups was neither accompanied by a decrease in the amount of oocysts shed in the feces, nor a marked drop in the density of parasites in the mucosa, further studies concerning the mechanism of action of NTZ and NTZ-R8 *in vivo* are highly needed. In general, the IFN- γ -knockout mouse model is considered appropriate for pre-clinical testing of anti-cryptosporidial drugs, however, it is essential to further evaluate the efficacy of NTZ-R8 in the natural host, e.g. neonate calves.

Unfortunately, the synthetic conjugate NTZ-R8 is not easily affordable. Consequently, the conjugate drug was only available in limited amounts and the test was performed at a rather low concentration of 2 mg/kg BW. For NTZ the regular dose would be 100-200 mg/kg BW [17, 23], but it was decided to apply a reduced treatment dose of NTZ 10 mg/kg BW to allow better comparison of NTZ-R8 and NTZ. We expected failure of the low dose NTZ treatment since even at a dose of 100 mg/kg BW, NTZ was demonstrated to fail in controlling the parasite infection [17]. However, the results obtained in the current study show in that high efficacy in decreasing oocysts excretion is achieved 24 h after treatment with a similar reduction by NTZ (96%) and NTZ-R8 (94%). Thereafter, the oocyst excretion was not that much reduced with oocyst counts averaging 60% of the values recorded for the sham treatment. A clear difference in terms of clinical health was seen in mice treated with NTZ-R8. Around 80% of mice treated with NTZ-R8 survived whereas this was the case for only 40% of NTZ treated mice, although it is not significant ($P > 0.05$). Although the treatment dose of NTZ-R8 was only 2 mg/kg BW, it demonstrated clinical efficacy even higher than that of a 5-fold dose of NTZ alone to avoid unnecessary animal suffering.

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It remains to be shown whether a dose-response relationship exists and whether a higher treatment dose of NTZ-R8 will result in even better efficacy or, on the contrary, higher toxicity. Whatsoever, it was observed that *C. parvum* induced weight loss was interrupted in surviving treated mice and weight even slightly increased at 12 dpi or 13 dpi in the NTZ-R8 group. Recovery of mucosal tissue required more time than expected, and the observation period should be extended in further studies to e.g. 21 dpi to evaluate how long it takes until complete regeneration is obtained in NTZ-R8 treated animals.

Altogether, octaarginine proved its suitability as drug delivery vehicle in cryptosporidiosis treatment in *in vitro* and *in vivo* studies. As a cell-penetrating peptide (CPP) octaarginine facilitates the uptake of NTZ even into intracellular parasite stages shielded by the membrane of the parasitophorous vacuole. This improves the efficacy of NTZ and probably reduces toxicity through dose reduction. Based on these findings, further studies should be encouraged including evaluation of NTZ-R8 in target species.

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Author contribution

T.N.H.B., A.D; F.K: Designed the study and wrote the manuscript with contributions of T.G and R.U; T.N.H.B, M.B and FK: established and optimized experimental protocols and data analysis; C.H and R.U performed histopathology examination.

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4. Comprehensive discussion

4.1 Developing a novel octaarginine-based transfection method for *Cryptosporidium parvum*

In general, transfection methods can be classified into two groups: viral and non-viral transfection (including physical or chemical). Every method has its advantages and disadvantages. For instance, the viral transfection is based on the ability of a virus to integrate into the host genome. Thus, the transfection efficiency is quite high using respective methods. However, the viral-mediated transfection causes cytotoxicity and is subject to immunogenicity issues (KIM and EBERWINE 2010). To avoid this, the non-viral transfection is either based on electroporation or on the utilization of polycationic polymer commonly used than the viral transfection to introduce foreign genes into the host cells. Cell-penetrating peptides (CPPs) such as octaarginine are well-known for their intrinsic property to penetrate almost all known biological membranes and even the blood-brain barrier (GUIDOTTI et al. 2017). Strikingly, the ability of CPPs to deliver cargo across biological membranes does not depend on the size of the molecule to be transported but rather on the permeability of the membrane to the CPPs alone (KAMENA et al. 2011). Octaarginine was shown to penetrate almost all tested biological membranes with only few exceptions such as the membranes of non-infected red blood cells and the parasitophorous vacuole membrane of *Toxoplasma gondii* (KAMENA et al. 2008, SPARR et al. 2013). No respective information has been published before with respect to *C. parvum*.

In the current project, the intact oocyst wall of *C. parvum* was shown to be impermeable to octaarginine. When FAM-octaarginine was incubated with intact oocysts, short-time excysted (STE) oocysts, freshly excysted sporozoites and infected host cells, it was observed that the peptide can only stain the wall of intact oocysts without penetrating inside oocysts. This is probably due to the complex trilaminar structure of the *Cryptosporidium* oocyst wall. The very rigid oocyst wall makes *Cryptosporidium* oocysts resistant to harsh environmental conditions and to many chemical disinfection protocols (LENDNER and DAUGSCHIES, 2014). However, octaarginine easily penetrated into the STE oocysts, sporozoites and even into intracellular stages where the parasite is embedded in the parasitophorous vacuole. The kinetics of FAM-octaarginine uptake depends on the biological membranes. For example, the uptake by STE oocysts and sporozoites occurred within 30 minutes, which was much faster compared to the uptake by intracellular stages (trophozoites and meronts) which took around one hour. These findings encouraged the evaluation of perspectives for a new transfection method within comparison to electroporation, less aggressive transfection protocol. Another point of interest was whether improvement of drug delivery can be obtained by combining octaarginine with anti-cryptosporidial drugs.

Although CPPs transport other molecules across cell membranes without displaying relevant toxicity, their ability to carry DNA for transfection is very poor (JEONG et al. 2016). This can be explained by the unstable linear structure, or the short length of the peptide which is not sufficient for plasmid condensation (KILK et al. 2005). Therefore, CPPs have been modified by conjugating them with different chemical substances such as polyallylamine, poly-L-lysine, polyethylenimine (PEI) that show better plasmid condensation properties (SABOURI-RAD et al. 2017). Specifically, PEI shows excellent plasmid condensation properties, and high efficiency in gene delivery (FISCHER et al. 1999, NEUBERG and KICHLER 2014, ZHOU et al. 2018). By forming the proton sponge, PEI protects foreign DNA from lysosomal degradation and results in increasing transfected gene expression (THOMAS et al. 2019). However, PEI is also known to induce high cytotoxicity and to decrease cellular metabolic activities (FLOREA et al. 2002, GODBEY et al. 2001).

Interestingly, the combination of PEI and CPPs does not only significantly reduce cytotoxicity of PEI but also increases gene delivery efficiency (MUNYENDO et al. 2012). This observation prompted us to consider the combination of PEI and octaarginine for transfection of *C. parvum*. In order to identify the appropriate concentration of PEI to be used for the transfection, a gel retardation assay was conducted. The gel retardation can help to establish the optimal ratio of PEI/plasmid complex. It is characterized by the N/P ratio in which N is the number of amino groups positively charged and P is the number of phosphate groups negatively charged. It was observed that the entire plasmid was completely condensed at the N/P ratio of 12 and above (PUBLICATION 1). Interestingly, with the addition of octaarginine to the PEI/plasmid complex, the optimal N/P ratio was seen at a lower value of 10, which indicates that octaarginine might also contribute to DNA condensation. This result supported the assumption that combining PEI and octaarginine may enhance transfection of *C. parvum*. Consequently, the complex of DNA/PEI/octaarginine was used for transfection of freshly excysted sporozoites, STE oocysts, or intact oocysts (see Figure. 3 and Figure. 4). Successful transfection was only found when freshly excysted sporozoites or STE oocysts were used while transfection failed when intact oocysts were used. These results demonstrate that during excystation the permeability of the parasite stages increases resulting in higher susceptibility to transfection in the presence of octaarginine, highlighting the crucial role of octaarginine in this process.

Comprehension discussion

Figure 3: Analysis of permeation of FAM-octaarginine into intact oocysts, STE oocysts and sporozoites, intracellular stages of *C.parvum*

Comprehension discussion

Figure 4: Summary of the transfection protocol for *C. parvum* using DNA/PEI/octaarginine

Interestingly, the transfection with STE oocysts yielded a higher amount of transgenic parasites in comparison to freshly excysted sporozoites where transgenic parasites could be rarely found. In spite of many attempts to increase transfection efficiency for excysted sporozoites, successful transfection was only occasionally achieved. Although long time incubation did not impair the survival of sporozoites in terms of continuing motility and integrity of morphology, it seemed to dramatically reduce the ability of the parasites to infect host cells, and thus obviously altered viability. The reason for higher susceptibility to transfection of oocysts that were exposed to excystation only for a short time (“STE oocysts”) in comparison to fully excysted sporozoites appears unclear so far and remains to be further investigated.

The new transfection protocol based on PEI and octaarginine presents several advantages compared to conventional electroporation. First, it requires a lower amount of DNA (1-5µg) than the electroporation protocol (40 µg) (POTTER and HELLER 2003). Secondly, the principle of electroporation is based on introducing a high voltage electric shock to leak the cell membrane thus enabling transfer of foreign DNA into host cells. With that process, a large number of parasites are insulted and even killed, thus it limits the yield of alive transfected organisms. Therefore, electroporation-based approaches require a high number of parasites of around 1×10^7 (PAWLOWIC et al. 2017). With the new method reported here 50 times less parasites 2×10^5 are required. However, transfection with either PEI and plasmid or octaarginine and plasmid alone did not yield any GFP expressing parasites (data not shown). Thus it is obvious that PEI and octaarginine are both essential components in the combination used for the newly developed transfection protocol. In the current study, the non-covalent conjugation was applied to combine octaarginine and PEI by mixing to form the desired chemical complex. The non-covalent conjugation method has been widely applied in previous studies, e.g. on the interaction between avidin and biotin (WIERZBICKI et al. 2014). A major advantage of the non-covalent method is that it is easy to perform without requiring any special chemicals, and is thus easily accessible for research. Altogether, the PEI/octaarginine- based transfection method is a highly suitable method for *Cryptosporidium* transfection requiring only small amounts of DNA and achievable numbers of parasites, and is simple to perform.

4.2 Octaarginine as a vehicle for nitazoxanide (NTZ) delivery

Although cryptosporidiosis has been recognized as an important public health problem causing morbidity and mortality not only in animals but also in man, there is a lack of effective treatment options in any host species. Vaccines are not available so far. Halofuginone is a licensed drug for prevention and treatment of cryptosporidiosis in calves in Europe (KEIDEL and DAUGSCHIES 2013). In man, NTZ [2-acetyloxy-N-(5-nitro-2-thiazolyl) benzamide] is currently the only drug approved by FDA for cryptosporidiosis treatment. However, NTZ shows the limited efficacy on cryptosporidiosis if applied

to persons being under particular risk such as immunocompromised people and malnourished children (CABADA and WHITE 2010, MEAD and ARROWOOD 2014). Disappointing results were also obtained by *in vivo* testing in immunocompromised mice (MANJUNATHA et al. 2017). Therefore, there is a pressing need for developing novel and efficient treatments and hopefully vaccines. Recently, several potential drug candidates for *Cryptosporidium* treatment were tested *in vitro* and *in vivo* such as CDPK1 inhibitor (LENDNER et al. 2014), oleyphosphocholine (SONZOGNI-DESAUTELS et al. 2015), bumped kinase inhibitor (SCHAEFER et al. 2016), PI (4) inhibitor (MANJUNATHA et al. 2017), clofazimine (LOVE et al. 2017), and benzoxaborole (LUNDE et al. 2019). However, the procedure for approving a new drug is cumbersome, costly and takes a long time. Alternative, other approaches such as drug repurposing or empowering of available drugs could be applied to achieve suitable treatment options in due course. One of the most crucial factors influencing the development of novel drugs is the delivery efficiency (FOGED and NIELSEN 2008). The host plasma membrane of the PV is the barrier that hinders the translocation of chemical substances to the site of *C. parvum* infestation (ZHANG et al. 2019). The host cell membrane protects the entire cell and regulates e.g. nutrient transport and metabolite excretion. Likewise, it also prevents the uptake of pharmaceuticals and especially hydrophilic drug molecules (RAUTIO et al. 2008).

NTZ is classified into the class IV group which is characterized by low solubility and low permeation across the membranes according to the Biopharmaceutical Classification System (FIRAKE et al. 2017). Due to the low solubility, NTZ has low bioavailability and it therefore, requires high doses to achieve sufficient efficacy (FÉLIX-SONDA et al. 2014). Most importantly, the balance between the efficacy at high dosage and related cytotoxicity requires attention. The general strategy to overcome solubility limitations is the generation of novel solid phases such as amorphous forms, solvates, salts or cocrystals (GADADE and PEKAMWAR 2016). In the current study, we have opted to enhance the permeability of cell membranes to NTZ by linking it to the CPP octaarginine.

CPPs have been widely applied in pharmaceuticals and examined for their capacity to overcome the plasma membrane barrier and other biological membranes without any indication of cytotoxicity (BECHARA and SAGAN 2013; DINCA et al. 2016). In the current study, NTZ was coupled to octaarginine to deliver NTZ to the intracellular site of parasite location. The results presented in PUBLICATION 1 and MANUSCRIPT 2 demonstrate the high permeability of *Cryptosporidium* membranes to octaarginine. In previous studies by SPARR et al. (2013), it was shown that coupling of the anti-malaria drug fosmidomycine to octaarginine considerably enhances the uptake of the drug and hence its efficacy for more than 40-fold as compared to fosmidomycine alone. This study also points out that octaarginine is harmless to host cells. Those results encouraged the hypothesis that higher efficacy of NTZ can be obtained through increase of its uptake after coupling to octaarginine.

NTZ was chosen for two reasons. Firstly, this is the only approved drug for cryptosporidiosis treatment in humans. Secondly, the chemical structure of NTZ is ideally suited for a reversible coupling to octaarginine. In general, the stability of CPPs and the capacity of the liberation of cargo are two major concerns related to applying CPPs for the delivery of pharmaceuticals. NTZ is a pro-drug that is activated by esterase inside the cells to generate the active molecule tizoxanide (FOX and SARAVOLATZ 2005). This offers the option to couple octaarginine to the labile ester function of NTZ as an esterase- releasable moiety (Figure 5). Normally, natural peptides are rapidly degraded when exposed to intestinal enzymes (KRISTENSEN et al. 2016). However, using a synthetic non-natural β -octaarginine derivative renders the peptide resistant to peptidase.

Figure 5: The chemical structures of tizoxanide, nitazoxanide (NTZ) and nitazoxanide-octaarginine (NTZ-R8). Red arrow showing the cleavage position by esterase to release tizoxanide from NTZ-R8

Testing anti-cryptosporidial candidate compounds *in vitro* is a crucial step in drug development. To perform *in vitro* testing on *C. parvum* intracellular stages, these have to be cultured in appropriate cell lines that originate from animal or human tissues. Attempts to apply axenic cultures did not result in success in terms of appropriate screening models. Particularly, the human ileocecal adenocarcinoma (HCT-8) cell line has been widely used in *Cryptosporidium* research. Up to now, many read-outs for evaluation of *in vitro* results have been reported, including microscopic counting of parasitic stages, enzyme-linked immunosorbent assay, chemiluminescence immunoassay (YOU et al. 1996), real-time quantitative PCR (SHAHIDUZZAMAN et al. 2009), or quantification of transgenic parasites expressing luciferase (VINAYAK et al. 2015). At present, measuring DNA of parasites by quantitative PCR is widely used because of high accuracy and convenience. The study of CAI et al. (2005) pointed out that under *in vitro* conditions, the decay of DNA of dead parasites (99% in 24 h to 48 h) took longer than that of RNA (99% in 3 h). Hence, the level of RNA was considered to better represent for live parasites than DNA assessment. In fact, the level of 18sRNA transcribed by parasites after exposure to anti-

cryptosporidial treatment allowed to detect only living parasites (CAI et al. 2005, ZHANG and ZHU 2015) and prevented errors in undermining drug efficacy. 18sRNA of host cells was applied for normalization. The advantage of the reverse-transcriptase PCR method is higher accuracy compared to quantitative PCR. However, the method bears a disadvantage since RNA is not as stable as DNA and thus the quantification may easily lead to false negative results.

The combination of NTZ and octaarginine (NTZ-R8) was tested for cytotoxicity before assessment of *in vitro* inhibition of *C. parvum*. According to ISO 10993-5, cell viability of more than 80% is considered to reflect non-cytotoxicity. In the current study, cell viability of cultures exposed to the highest concentration of NTZ (25 µg/ml) and octaarginine (100 µg/ml) was 85% and 99%; respectively. Thus, the used concentrations were all in the range of non-cytotoxicity. These results were compatible to those obtained by SHARMA et al. (2014). Serial dilutions of NTZ and NTZ-R8 (1000, 100, 50, 10, 5, 1 ng/ml) were tested in a growth inhibition assay of *C. parvum*. The results reported in MANUSCRIPT 2 demonstrate that NTZ coupled to octaarginine (NTZ-R8) dramatically improved the growth inhibition of *C. parvum* within 24 h post exposure. The IC₅₀ value was 60.54 ng/ml (197 nM) for uncoupled NTZ while IC₅₀ of NTZ-R8 was much lower with only 4.499 ng/ml (2.9 nM). As expected, octaarginine supported passage of other molecules through cell membranes, and NTZ coupled with octaarginine easily penetrated into the parasite, even when the parasites were located in the parasitophorous vacuole. In summary, the aim to achieve improved the inhibition of the parasite was perfectly reached under *in vitro* conditions.

In current study, 1000 ng/ml of NTZ inhibited *C. parvum* development by 79%, and thus anti-cryptosporidial efficacy was seen at a far higher than reported in previous publications (GRIFFITHS et al. 1998, CAI et al. 2005, SHARMA et al. 2014). In general, NTZ has a low aqueous solubility (7.5 ng/ml) (SALAS-ZÚÑIGA et al. 2020). Therefore, choosing an appropriate solvent for NTZ is very crucial for *in vitro* studies. Although NTZ dissolves in DMSO, precipitation was observed when NTZ was further diluted in culture medium DMEM. To tackle this problem, a short exposure of NTZ in DMEM to sonication for 30 seconds (2-3 times) appeared to be a suitable procedure to increase solubility. The sonication was applied to dissolve both NTZ and NTZ-R8. Both the developed protocol to increase solubility and the highly sensitive evaluation method (RT-qPCR) may explain why the IC₅₀ observed for NTZ was lower in the current evaluation than in previous studies.

Several anticoccidial drugs were shown before to control *Cryptosporidium* under *in vitro* conditions (SHAHIDUZZAMAN et al. 2009, SHARMA et al. 2014). However, only few compounds such as halofuginone and PRM present a partial inhibitory activity on *Cryptosporidium* infection in animal models (MEAD and ARROWOOD 2014). There are many elements such as bioavailability, pharmacokinetics, and food-drug interaction (KOZIOLEK et al. 2019) which may explain the

difficulties in concluding on efficacy of a drug based on *in vitro* testing only (SONZOGNI-DESAUTELS et al. 2015).

To perform *in vivo* drug testing for *Cryptosporidium* infection, it is crucial to choose an appropriate animal model. In the current study, the IFN- γ -knockout mouse model was used because it is considered to be highly susceptible to cryptosporidiosis (OETTINGEN et al. 2008, SONZOGNI-DESAUTELS et al. 2015) and to at least partly mimic the response of immunocompromised patients to infection with *Cryptosporidium*. Other mouse models such as dexamethasone-treated mice (RASMUSSEN and HEALEY 1992), severe combined immunodeficiency (SCID) mice (TZIPORI et al. 1995), neonate mice (DOWNEY et al. 2008, DELAUNAY et al. 2000) were also applied with more or less success. However, all of these mouse models required a high amount of oocysts ranging from 10^5 - 10^6 per animal for infection. In contrast, C57BL/6 IFN- γ - knockout mice display severe illness following inoculation of only 10 oocysts (OETTINGEN et al. 2008). Furthermore, IFN- γ - knockout mice can be suitable for analyzing the efficacy of drug therapy on acute infection at the reasonable infection doses (YOU and MEAD 1998, GRIFFITHS et al. 1998, SONZOGNI-DESAUTELS et al. 2015). Therefore, such mice were assumed ideal to confirm the promising *in vitro* data on the anti-cryptosporidial properties of NTZ-R8 in a laboratory animal model. Altogether, the IFN- γ - knockout mouse model is considered appropriate for pre-clinical testing of anti-cryptosporidial drugs.

The infection doses tested in the current experiments (MANUSCRIPT 2) were 1000, 5000, and 10000 oocysts per mouse. Regardless of how many oocysts were used for the infection, the peak of infection was recorded at 8 dpi. However, the prepatency differed between the groups of mice. Infection with 10000 oocysts resulted in patency starting 1 dpi while the earliest excretion was seen on 2 dpi in mice infected with lower infection dose of 1000 or 5000 oocysts. The severity of sickness did not depend on the number of inoculated oocysts. Our findings were compatible with the results from GRIFFITHS et al. (1998) obtained in the INF- γ knock mouse model, and also of data obtained in other mouse models (YANG et al. 2000, ZHENG 2020). Consequently, the infection dose of 1000 oocysts was deemed sufficient and chosen for further *in vivo* trials. This inoculation dose was described to be lethal to mice between 8 dpi and day 14 dpi (YOU and MEAD 1998). Therefore, it was decided to treat infected mice at 6 dpi order to assess protection achieved by treatment in terms of clinical disease. In fact, typical clinical symptoms of cryptosporidiosis such as weight loss, anorexia, dehydration, and ruffled fur were observed and considerable oocyst excretion was seen in infected IFN- γ knockout mice. The histopathological examination revealed typical pathogenic lesions as villous atrophy, crypt hyperplasia and degenerating epithelial cells within the crypt lumina. However, the most typical symptom of cryptosporidiosis in infected neonate calves (KEIDEL and DAUGSCHIES 2013, THOMSON et al. 2017, AYDOGDU et al. 2018) and in humans (DAVIES and CHALMERS 2009, REHN et al. 2015) is diarrhoea, which cannot be reproduced in this mouse model. In general, lacking diarrhoea has been

reported as one of the drawbacks of mouse models in cryptosporidiosis research (GRIFFITHS et al. 1998). Consequently, we could not demonstrate efficacy of NTZ or NTZ-R8 on *C. parvum* induced diarrhoea. However, since cryptosporidiosis was well controlled by NTZ-R8, it appears highly probable that a distinct effect on diarrhoea will be seen if NTZ-R8 is applied in the target species. This assumption has to be investigated in future studies.

Because of the very high expense associated with the chemical synthesis of NTZ-R8, only treatment of one group with a low concentration of 2 mg/kg BW could be tested in this study. Since it was expected that coupling to octaarginine would dramatically increase NTZ efficacy, we decided to compare treatment efficacy to that of a five-fold higher dose of NTZ (10 mg/kg BW) alone. In other studies, a much higher dose of NTZ of 100-200 mg/kg BW was used in mice (THEODOS et al. 1998, MANJUNATHA et al. 2017). Even a very high NTZ dose as 100 mg/kg BW showed only modest efficacy on the parasite (MANJUNATHA et al. 2017). Therefore, it was expected that a low dose of 10 mg/kg BW might fail to prevent disease as in fact observed in the experiment. Inclusion of an additional sham treatment group with an even lower dose of such as 2 mg/kg BW (the dose used for NTZ-R8) would most probably have caused additional and serve animal suffering without contributing to extension of knowledge.

The results of oocysts shedding obtained in current study showed that high efficacy in reducing number of oocysts was observed after 24 h treatment with a similar reduction by 10 mg/kg BW NTZ (96%) and 2 mg/kg BW NTZ-R8 (94%). Thereafter, OpG values were reduced to around 60% in both treatment groups as compared to sham treatment. However, the survival rate and general health conditions (smooth fur, activity, posture, and capacity to response) of mice in both treated groups were generally superior compared to the sham treated mice. The latter displayed ruffled fur, trembling and delayed response to stimulation at 9 dpi. Altogether, both NTZ and NTZ-R8 dramatically enhanced performance of infected mice. While all infected mice in the shame treated group died at 11 dpi, treated mice survived until the end of the experiment. Remarkably, NTZ-R8, although given at a low treatment dose of only 2 mg/ kg BW, displayed a higher efficacy than NTZ alone at 5-fold higher dose (10 mg/kg BW) by allowing survival of 80% of the respective mice in comparison with 40% of mice that were treated with NTZ only. Thus, octaarginine improved dramatically the efficacy of NTZ treatment of *Cryptosporidium* infection, especially in terms of survival while OpG values were less affected.

Although the current study demonstrates the suitability of octaarginine as a vehicle to deliver NTZ across membranes and thus opens new options to improve *Cryptosporidium* treatment, further investigations are necessary. For instance, dose titration studies in further laboratory rodent experiments are pivotal to define the most appropriate dose of NTZ-R8. Besides that, the length of infection experiments should be extended to 21 days or even longer to better understand long term effects of

treatment on cryptosporidiosis, e.g. in terms of weight development or histopathological alterations of the ileum.

It is obvious that immunodeficient mice are a rather artificial model. Results obtained from respective studies are though important in terms of pre-clinical screening, to validate *in vitro* findings and to construct and evaluate theoretical considerations. However, this does not replace experiments in natural hosts such as calves for *C. parvum*. In fact, the suitability of NTZ-R8 clearly remains to be demonstrated in the target species and cannot be postulated based on the current *in vitro* and *in vivo* data obtained from laboratory rodent experiment alone. Moreover, aspects like toxicity, pharmacodynamics and side effects have to be addressed, particularly if NTZ-R8 is considered as a drug for use in livestock or in humans.

5. Conclusion

The combination of octaarginine and PEI- two polycationic molecules well suited for the delivery of plasmid DNA into *Cryptosporidium*. It is important to note that successful transfection depends on the permeability of the parasite membrane to octaarginine albeit not exclusively, since free sporozoites were poorly transfected although they show the highest permeability to octaarginine. A limitation of using sporozoites in transfection is probably rapid loss their infectivity during various incubation steps. In contrast, short time excystation (STE) oocysts yield the best results because in addition to being permeable to octaarginine and sporozoites of STE oocysts are exposed to excystation and transfection medium for a short period rendering them more vital than fully excysted oocysts. The major advantages of our novel transfection protocol are that it requires a very low amount of plasmid DNA is simple to perform and does not require any sophisticated equipment. In addition, the capacity of octaarginine to function as drug delivery vehicle in *Cryptosporidium* treatment was also clearly established. Coupling of NTZ to octaarginine (NTZ-R8) dramatically improved treatment efficacy at a distinctly reduced dose of the active ingredient. These findings usher in new paths for treatment of *Cryptosporidium* infection in animals and in human.

6. Summary

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Studies into the suitability of the cell-penetrating peptide octaarginine as a transmembrane vehicle for DNA transfection of *Cryptosporidium parvum* and to improve the antiprotozoan efficacy of Nitazoxanide

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Key words: *Cryptosporidium parvum*, octaarginine, transfection, PEI, nitazoxanide, nitazoxanide-octaarginine, INF- γ knockout mice

Introduction: *Cryptosporidium parvum* is one of the most common causes of diarrhea worldwide in neonatal calves. This pathogen is also life-threatening in malnourished children and immunodeficient patients. There is no vaccine and a single drug nitazoxanide (NTZ), of only the moderate efficacy has been approved by FDA for cryptosporidiosis treatment in human. Octaarginine is known to facilitate the transport of other molecules across cell membranes and has been used to transfect protozoan organisms. It is also proposed to increase the efficacy of drugs against intracellular pathogens.

Aims of the study: The capacity of octaarginine to support transfection of *C. parvum* as an alternative to electroporation was evaluated. Furthermore, it was studied whether octaarginine covalently bound to NTZ (NTZ-R8) improves efficacy against the parasite.

Animals, materials and methods: FAM-octaarginine was added to either intact oocysts, short-time excystation exposed (STE) oocysts, excysted sporozoites, intracellular stages of *C. parvum* to assess the permeability of the *Cryptosporidium* membrane to the peptide. The optimal conditions for condensation of plasmid for transfection experiments were evaluated by testing different N/P ratios applying by gel retardation assay. The transfection complex octaarginine/polyethyleneimine (PEI)/DNA was also incubated with intact oocysts, STE oocysts, and excysted sporozoites. Transfected parasites were transferred to HCT-8 cell cultures and further incubated for 24 h. Immunofluorescence assay (IFA) was performed to detect successfully transfected parasites. To evaluate the suitability of octaarginine as a vehicle supporting transport of NTZ across membranes, octaarginine was coupled to NTZ to produce NTZ-R8. *Cryptosporidium* oocysts were inoculated into HCT-8 cell monolayers in the presence of NTZ and NTZ-R8 at concentrations of 1, 5, 10, 50, 100 or 1000 ng/ml. Parasite growth was monitored by RT-qPCR after RNA extraction from *C. parvum* exposed HCT-8 cell cultures. RT-qPCR was performed

Summary

on the target gene 18S rRNA of *Cryptosporidium* and normalized to the expression of the housekeeping gene 18S rRNA of host cells. To evaluate the efficacy of NTZ-R8 *in vivo*, IFN- γ knockout mice were orally inoculated with 1000 oocysts each, except for the non-infected controls. Infected mice were treated with NTZ (10 mg/kg BW) or NTZ-R8 (2 mg/kg BW) in 7 days. The efficacy of treatment was evaluated by oocyst excretion, survival rate, clinical symptoms, and histopathological changes in the ileum.

Results: Octaarginine easily penetrated into *Cryptosporidium* sporozoites and STE oocysts, and intracellular stages while the membrane of intact oocysts remained impermeable. The optimal N/P ratio for the full DNA plasmid condensation starts from 10 when octaarginine was also added to the complex. Successful transfection of excysted sporozoites and STE oocysts was observed with only 1 μ g plasmid in the transfection complex. Transfection was not achieved when intact oocysts were used. The half-maximal inhibition concentration (IC₅₀) of NTZ and NTZ-R8 was 60.54 ng/ml (197 nM) and 4.499 ng/ml (2.9 nM), respectively. Therefore, octaarginine significantly improved inhibition *C. parvum* growth by NTZ around 68 times ($P < 0.05$). During *in vivo* studies, it was observed that infected mice displayed symptoms of cryptosporidiosis such as anorexia, weight loss and ruffled fur. Mice treated with NTZ at 10 mg/kg BW displayed in 40% survival while mice treated with NTZ-R8 at 2 mg/kg BW showed a distinctly higher survival rate of 80%, albeit non-significant ($P > 0.05$).

Conclusion: DNA condensation by PEI and DNA delivery by octaarginine allows simple and rapid transfection that requires a small amount of plasmid DNA only and does not depend on sophisticated equipment. Best results were obtained using STE oocysts. Octaarginine also successfully transported the anticryptosporidial compound NTZ into extracellular and intracellular stages of *C. parvum* and is therefore a suitable vehicle for drug delivery, thus being a promising tool for improvement of treatment efficacy.

7. Zusammenfassung

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Untersuchungen zur Eignung des zellpenetrierenden Peptids Octaarginin als Transmembranvehikel für die DNA-Transfektion von *Cryptosporidium parvum* und zur Verbesserung der antiprotozoären Wirksamkeit von Nitazoxanid

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Schlüsselwörter: *Cryptosporidium parvum*, Octaarginin, Transfektion, PEI, Nitazoxanid, Nitazoxanid-Octaarginin, INF- γ -Knockout-Mäuse

Einleitung: *Cryptosporidium parvum* ist weltweit eine der häufigsten Ursachen für Durchfallerkrankungen bei neugeborenen Kälbern. Auch für unterernährte Kinder und immungeschwächte Patienten ist dieser Erreger lebensbedrohlich. Es gibt keinen Impfstoff, und ein einziges Medikament von mäßiger Wirksamkeit, Nitazoxanid (NTZ), wurde von der FDA für die Kryptosporidiose-Behandlung beim Menschen zugelassen. Octaarginin erleichtert den Transport von Molekülen durch Zellmembranen und kann zur Transfektion von Protozoen verwendet werden. Es wird vermutet, dass es auch die Wirksamkeit von Medikamenten gegen intrazelluläre Krankheitserreger erhöhen kann.

Ziele der Untersuchungen: Die Eignung von Octaarginin, *C. parvum* zu transfizieren wurde als Alternative zur Transfektion mittels Elektroporation untersucht. Darüber hinaus wurde getestet, ob kovalent an NTZ gebundenes Octaarginin dessen Wirksamkeit gegen den Parasiten steigert.

Tiere, Material und Methoden: FAM-markiertes Octaarginin wurde entweder intakten Oozysten, teil-exzystierten (STE) Oozysten, exzystierten Sporozoiten, oder intrazelluläre Stadien von *C. parvum* zugesetzt, um die Permeabilität der Kryptosporidienmembran für das Peptid zu bewerten. Die optimalen Bedingungen für die Plasmidkondensation zur Transfektion wurden durch Testen verschiedener N/P-Verhältnisse untersucht und durch einen Gelretardierungstest festgelegt. Der Transfektionskomplex wurde mit intakten Oozysten, STE-Oozysten und exzystierten Sporozoiten inkubiert. Transfizierte Parasiten wurden auf HCT-8-Zellkulturen übertragen und 24 h weiter inkubiert. Zum Nachweis erfolgreich transfizierter Parasiten wurde ein Immunfluoreszenztest (IFA) durchgeführt. Um die Eignung von Octaarginin als Vehikel für den Transport von NTZ über Zellmembranen zu analysieren,

wurde NTZ-R8 durch Koppelung von Octaarginin an NTZ produziert. *Cryptosporidium*-Oozysten wurden auf HCT-8-Zell-Monolayern in Gegenwart von NTZ und NTZ-R8 in Konzentrationen von 1 bis 1000 ng/ml) gegeben. Das Parasitenwachstum wurde mittels RT-qPCR nach RNA-Extraktion aus *C. parvum*-exponierten HTC-8-Zellkulturen anhand des *C. parvum* Zielgens 18S rRNA durchgeführt. Die Expression des 18S rRNA-Gens der Wirtszellen diente der Normalisierung. Um die Wirksamkeit von NTZ-R8 in vivo zu bewerten, wurden IFN- γ Knockout-Mäuse oral mit jeweils 1000 Oozysten infiziert. Die Mäuse wurden 7 Tage *post infectionem* (dpi) mit NTZ-R8 (2 mg/kg KGW) oder NTZ in einer fünffach höheren Dosis (10 mg/kg KGW) behandelt. Die Wirksamkeit der Behandlung wurde vergleichend anhand der Oozystenausscheidung, der Überlebensrate, der klinischen Symptome und histopathologischer Befunde im Ileum bewertet.

Ergebnisse: Über die FAM-Markierung konnte dargestellt werden, dass Octaarginin leicht in *Cryptosporidium*-Sporozysten und STE-Oozysten sowie in intrazelluläre Stadien des Erregers eindringt, während die Wand intakter Oozysten völlig undurchlässig ist. Das optimale N/P-Verhältnis für die vollständige DNA-Plasmidkondensation liegt in Anwesenheit von Octaarginin bei einem Wert von 10. Eine erfolgreiche Transfektion von exzystierten Sporozysten und STE-Oozysten wurde mit nur 1 μ g Plasmid-DNA erreicht, während intakte Oozysten nicht transfiziert werden konnten. In HCT-8-Monolayern lag der IC₅₀-Wert von NTZ bei 60,54 ng/ml (197 nM), während er mit NTZ-R8 deutlich auf 4,499 ng/ml (2,9 nM) verringert werden konnte und die In-vitro-Wirksamkeit von NTZ durch Koppelung an Octaarginin um das 68-fache signifikant ($P < 0,05$) gesteigert wurde. In den In-vivo-Studien zeigten infizierte Mäuse Anorexie, Gewichtsverlust und struppiges Fell. Mit 10 mg NTZ/kg KGW behandelte Mäuse hatten eine Überlebensrate von 40 %, während mit 2 mg NTZ-R8/kg KGW behandelte Mäuse eine deutlich höhere Überlebensrate von bis zu 80 % aufwiesen, dieser Effekt war allerdings nicht signifikant ($P > 0,05$).

Schlussfolgerung: Eine neue Methode zur einfachen und schnellen Transfektion von *C. parvum*, die nur eine kleine Menge Plasmid-DNA erfordert und nicht von einer hochmodernen Ausstattung abhängt, wurde entwickelt. Die besten Ergebnisse wurden bei der Verwendung von STE-Oozysten erzielt, während intakte Oozystenwände nicht permeabel sind. Octaarginin transportierte NTZ in extrazellulär und intrazelluläre Stadien von *C. parvum* und erwies sich *in vitro* wie *in vivo* als ein geeignetes Vehikel für den Transport von NTZ in den Erreger, was sich in einer klaren Verbesserung der Behandlungseffizienz spiegelte.

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