A novel CDPK1 inhibitor—a potential treatment for cryptosporidiosis in calves?

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Abstract *Cryptosporidium parvum* is a zoonotic agent that infects humans and animals occasionally causing severe, watery diarrhoea. In immunocompetent hosts, cryptosporidiosis is self-limiting but can have a fatal outcome in immunocompromised individuals. *Cryptosporidium* is one of the most common causes of waterborne diseases (recreational water and drinking water) in humans, a leading cause of moderate to severe childhood diarrhoea, and a major agent of diarrhoea in calves leading to high economic losses and up to 10 % lethality. So far, available treatment options are insufficient for both veterinary and human clinical disease cases. Here, we report for the first time that the novel bumped kinase inhibitor (BKI) 1294 targeting the calcium-dependent protein kinase 1 (CDPK1) of *Cryptosporidium* is able to reduce the oocyst shedding of *C. parvum* by calves—its natural host—without obvious side effects.

Keywords *Cryptosporidium parvum* · Cryptosporidiosis · Treatment · CDPK1 · Bumped kinase inhibitor

Cryptosporidium parvum is a unicellular, zoonotic agent that infects a wide range of hosts. In humans, cattle and goats, it causes severe watery diarrhoea which is often self-limiting but can also have a fatal outcome. In young calves, *C. parvum* is the most common cause of diarrhoea affecting nearly every

animal (Göhring et al. 2014). Consequently, calves are supposed to be a major source of oocysts infecting humans especially in rural areas with poor sanitation and close contact between humans and livestock. A recent global enteric multi-center study (GEMS) identified *Cryptosporidium* sp. as the second most common pathogen in children with moderate to severe diarrhoea, and *Cryptosporidium* was significantly associated with an increased risk of death of 12- to 23-month-old children (Kotloff et al. 2013).

Unfortunately, the treatment options for *Cryptosporidium* infections are limited. There are only two approved products on the market which are moderately effective against *Cryptosporidium*. Nitazoxanide is approved in the USA to treat humans, and halofuginone is approved in Europe for the treatment of calves (Lendner et al. 2011).

Calcium-dependent protein kinases (CDPKs) are plant-derived kinases which differ from mammalian kinases in having an enlarged adenosine triphosphate (ATP) binding pocket that is selectively inhibited by bumped kinase inhibitors (BKIs), such as 1294 (Castellanos-Gonzalez et al. 2013). *CpCDPK1* has an orthologue in *Toxoplasma gondii* which was shown to play a crucial role in invasion (Lourido et al. 2010). Consequently, BKIs have been developed to prevent invasion by inhibiting CDPK function (Lourido et al. 2010; Ojo et al. 2010; Johnson et al. 2012). In *T. gondii*, it has been shown that BKI 1294 selectively targets through *TgCDPK1* by overexpressing a mutant form of *TgCDPK1*. Mutant *T. gondii* displayed decreased sensitivity to BKI 1294 (Ojo et al. 2010). It was shown that BKI 1294 is a potent inhibitor of *CpCDPK1* and prevents the invasion of host cells by *C. parvum* in vitro and the establishment of the disease in mice (Castellanos-Gonzalez et al. 2013). However, *C. parvum* does not infect immunocompetent mice making the transfer of results from experiments using immunosuppressed mice to the natural host difficult. Here, we demonstrate for the first time that BKI 1294 reduces *C. parvum* oocyst shedding in its natural host.

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Table 1 Oocyst shedding and reduction in shedding of the BKI 1294 group compared to the other groups; shown is the sum of oocysts shed by each group and per animal

Group	No. of animals	Σ Oo per field of vision and group	Σ Oo per field of vision and animal	Percent reduction	Mean no. of Oo per field of vision
BKI1294	6	38.13	6.40		0.6348
Mock	6	155.75	25.96	75.35	2.584 ($p=0.0008$)*
Untreated	5	98.18	19.64	67.41	2.039 ($p=0.0232$)*

Oo oocyst

*ANOVA ($p \leq 0.05$) and Mann-Whitney test. p values indicate significance to the BKI1294 group

Three groups ($n=6$) of newborn calves were infected with a single dose (2×10^7) of *C. parvum* and were orally treated five times every 2 days with 400 mg/animal BKI 1294, mock control (solvent; 70 % ethanol, 30 % DMSO) or milk replacer starting 1 h after infection. Oocyst output (semiquantitative analysis according to Keidel and Dauschies 2013) and health status were assessed daily in this double-blinded study. The average oocyst output per animal per field of vision was calculated by summing up the oocyst output of each group for study days 4 to 14 and dividing it by the number of animals per group.

The single dose infection with *C. parvum* led to mild symptoms and only a few days with watery diarrhoea in all groups. In the untreated group, one animal (excluded) died on study day 6 due to a bacterial infection and one animal of the treatment group died on study day 17 due to an omphalogenic systemic infection associated with a patent urachus. BKI 1294-treated animals only showed a single peak of oocyst excretion at day 5 p.i., whereas the control groups showed a second much higher peak at day 8 p.i. The overall reduction in oocyst shedding in the BKI 1294 group was 76 % compared to the mock control group and 68 % compared to the untreated group (see Table 1).

This study shows that BKI 1294 might have a potential to treat cryptosporidiosis in calves and potentially in humans as well. However, only minor differences in faecal consistency and dehydration could be observed between the treatment group and the control groups. This is due to the fact that the infection with *C. parvum* in general was moderate in terms of diarrhoea and dehydration even in the controls. Seven of the animals, in one of the two cohorts of this experiment, had bloody faeces, which is unusual for *C. parvum* mono-infection, making it likely that the animals were infected with undetected enteropathogens. Since severe cryptosporidiosis in calves may reflect a multifactorial aetiology (Göhring et al. 2014), we analysed the faecal samples for other common enteropathogens such as rotavirus, coronavirus, *Escherichia coli* K99 and *Giardia* with commercially available fast tests (rotavirus, coronavirus, *E. coli* K99 with strip tests (Bio X diagnostics), *Giardia* with an ELISA (ProSpecT™)). The samples were negative for those pathogens. However, we cannot exclude the presence of other enteropathogens such as *Clostridium perfringens* that can cause enteritis. However,

further studies are needed to evaluate if BKI 1294 would reduce symptoms in a setting where animals develop long lasting (>5 days) moderate to severe diarrhoea without co-infections. Since cryptosporidiosis is a multifactorial disease in which severe symptoms occur predominantly in co-infected animals (Göhring et al. 2014), it would also be necessary to test if BKI 1294 would lead to an improvement with co-infections.

Altogether, the study shows that BKI 1294 could have the potential to treat cryptosporidiosis in calves and might also be a candidate to treat humans. The specificity of BKI 1294 for targeting *CpCDPK1*, and not human protein kinases (Castellanos-Gonzalez et al. 2013), makes it highly probable that no relevant side effects would occur in humans, as it was the case for calves in this experiment.

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