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Safety and efficacy of the bumped kinase inhibitor BKI-1553 in pregnant sheep experimentally infected with *Neospora caninum* tachyzoites

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ABSTRACT

Neospora caninum is one of the main causes of abortion in cattle, and recent studies have highlighted its relevance as an abortifacient in small ruminants. Vaccines or drugs for the control of neosporosis are lacking. Bumped kinase inhibitors (BKIs), which are ATP-competitive inhibitors of calcium dependent protein kinase 1 (CDPK1), were shown to be highly efficacious against several apicomplexan parasites *in vitro* and in laboratory animal models. We here present the pharmacokinetics, safety and efficacy of BKI-1553 in pregnant ewes and foetuses using a pregnant sheep model of *N. caninum* infection. BKI-1553 showed exposure in pregnant ewes with trough concentrations of approximately 4 μM, and of 1 μM in foetuses. Subcutaneous BKI-1553 administration increased rectal temperatures shortly after treatment, and resulted in dermal nodules triggering a slight monocytosis after repeated doses at short intervals. BKI-1553 treatment decreased fever in infected pregnant ewes already after two applications, resulted in a 37–50% reduction in foetal mortality, and modulated immune responses; IFNγ levels were increased early after infection and IgG levels were reduced subsequently. *N. caninum* was abundantly found in placental tissues; however, parasite detection in foetal brain tissue decreased from 94% in the infected/untreated group to 69–71% in the treated groups. In summary, BKI-1553 confers partial protection against abortion in a ruminant experimental model of *N. caninum* infection during pregnancy. In addition, reduced parasite detection, parasite load and lesions in foetal brains were observed.

1. Introduction

Neospora caninum (Apicomplexa: Eimeriina: Sarcocystidae) is an obligate intracellular parasite, known to be one of the most important infectious causes of abortion in cattle worldwide (Dubey and Schares, 2011; Dubey et al., 2017). Since its discovery, *N. caninum* has been identified in various species of livestock, including cattle, sheep, goats, horses and deer (Dubey et al., 2007). Cattle can become infected by horizontal transmission via the ingestion of oocysts, or by vertical transmission (i.e., transplacentally) as a result of either a primary infection of the dam by oocysts (exogenous transplacental transmission) or recrudescence of a chronic infection (endogenous transplacental transmission) during pregnancy, with different clinical and epidemiological consequences (Williams et al., 2009).

The clinical and economic importance of neosporosis in small ruminants has historically been considered much less relevant compared to infection by *Toxoplasma gondii*, which is one of the most common causative agents of abortion in sheep and goats (Dubey, 2009). However, recent evidence suggests that *N. caninum* is also an important abortifacient in small ruminants (Moreno et al., 2012) and may even be

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Table 1
Experimental design.

Group	Number of pregnant ewes	Number of foetuses/lambs	Inoculum (i.v.)	Treatment (s.c.)
G1	8	14	Nc-Spain7 10^6 tachyzoites	BKI-1553, 1st dose: 35 mg/kg bodyweight; a week later, a 2nd dose at 10 mg/kg bodyweight
G2	5	7	PBS	BKI-1553, 1st dose: 35 mg/kg bodyweight; a week later, a 2nd dose at 10 mg/kg bodyweight
G3	8	13	Nc-Spain7 10^6 tachyzoites	BKI-1553, 7 doses at 10 mg/kg bodyweight every other day
G4	5	9	PBS	BKI-1553, 7 doses at 10 mg/kg bodyweight every other day
G5	8	13	Nc-Spain7 10^6 tachyzoites	None
G6	3	5	PBS	None

i.v.: intravenous route.
s.c.: subcutaneous route.

the main cause of reproductive losses in some flocks (West et al., 2006; González-Warleta et al., 2014). Experimental infections in pregnant sheep (McAllister et al., 1996; Buxton et al., 1998; Weston et al., 2009; Arranz-Solís et al., 2015) have shown that they are highly susceptible, and as in cattle, abortion and vertical transmission are the main consequences of infection.

Many control measures have been proposed to reduce *N. caninum* infection in cattle, including embryo transfer, artificial insemination of seropositive dams, culling of infected animals and replacement by healthy heifers, drug treatment and vaccination (Dubey et al., 2007). The latter two options have been identified as economically viable, provided suitable targets and efficacious drugs can be made available (Häsler et al., 2006a, b). Although experimental studies have revealed potent effects of several drugs *in vitro* and in laboratory animal models (Müller and Hemphill, 2011; Hemphill et al., 2016), only triazinon derivatives, such as ponazuril (Kritzner et al., 2002) and toltrazuril (Haerdi et al., 2006; Syed-Hussain et al., 2015), and the polyether ionophore antibiotic monensin (Vanleeuwen et al., 2011) have been tested in ruminants experimentally infected with *N. caninum,* but results remained ambiguous. To date, pregnant ruminant models of neosporosis have not been used for assessments of drug efficacy against *N. caninum* infection and vertical transmission.

Anti-parasitic drug development based on targeting kinase enzymes is a well-established approach (Rotella, 2012). Calcium dependent protein kinase 1 (CDPK1) represents a promising drug target, as CDPK1 is encoded by the apicoplast DNA, and is thus absent from mammalian hosts (Lourido et al., 2010; Murphy et al., 2010; Ojo et al., 2010). CDPK1 activity is essential for microneme secretion, host cell invasion, and egress of *T. gondii* (Kieschnick et al., 2001; Lourido et al., 2010) and can be effectively targeted by a class of ATP-competitive compounds, collectively named bumped kinase inhibitors (BKIs).

BKIs have a broad-spectrum activity that affects many apicomplexan parasites (Van Voorhis et al., 2017). BKI-1294, BKI-1517 and BKI-1553 were all effective against *N. caninum in vitro* and strongly interfered with transplacental transmission in a pregnant mouse model of neosporosis (Ojo et al., 2014; Winzer et al., 2015; Müller et al., 2017a). BKI-1553 has been developed based on a variant on the naphthalinyl-pyrazolopyrimidine scaffold of BKI-1294. BKI-1553 is highly efficacious against *T. gondii in vitro*. It exhibits a low human ether-a-go-go-related gene (hERG) ion channel inhibition, excellent systemic exposure, crosses the blood-brain barrier in mice when administered orally, and BKI-1553 treatment lead to reduced parasite burden in the brain, lungs and liver of *T. gondii* infected mice (Vidadala et al., 2016). We here report on the safety and efficacy of BKI-1553 treatment in pregnant sheep experimentally infected with *N. caninum* tachyzoites at mid-gestation, drug levels in foetuses, and its impact on vertical transmission.

2. Materials and methods

2.1. Ethics statement

All protocols involving animals were approved by the Animal Welfare Committee of the Community of Madrid, Spain, following proceedings described in Spanish and EU legislation (PROEX 166/14 -experiment 1- and PROEX 064/15 -experiment 2-, Law 32/2007, R.D. 53/2013, and Council Directive, 2010/63/EU). All animals used in this study were handled in strict accordance with good clinical practices, and all efforts were made to minimize suffering.

2.2. Experiment 1: pharmacokinetics, safety and efficacy of BKI-1553 in a pregnant sheep model of neosporosis

2.2.1. Animals and experimental design

Fifty-four pure Rasa Aragonesa breed female lambs aged 3 months were selected from a commercial flock. All animals were seronegative for *T. gondii*, *N. caninum*, Border disease virus (BDV), Schmallenberg virus (SBV), *Coxiella burnetii* and *Chlamydia abortus* as determined by enzyme linked immunosorbent assay (ELISA). Animals were maintained in isolation in Zaragoza University (Spain) facilities until 12 months of age. They were oestrus-synchronized and mated with pure-breed Rasa Aragonesa tups for 2 days, after which the rams were removed from the ewes. Pregnancy and foetal viability were confirmed by ultrasound scanning (US) on day 40 post-mating, and thirty-seven pregnant sheep were selected for the experiment. Pregnant ewes (n = 37) were randomly distributed into six experimental groups (see Table 1) and housed at the Clinical Veterinary Hospital facilities (Complutense University of Madrid, Spain). Twenty-four ewes were allocated into groups 1 (G1; n = 8), 3 (G3; n = 8) and 5 (G5; n = 8), which were inoculated intravenously with 10^6 tachyzoites of the bovine isolate Nc-Spain7 (Regidor-Cerrillo et al., 2008) at day 90 of gestation (dg). The thirteen remaining pregnant ewes were allocated to groups 2 (G2; n = 5), 4 (G4; n = 5) and 6 (G6; n = 3), which received an intravenous inoculum of phosphate-buffered saline (PBS) at 90 dg.

BKI-1553 was synthesized by Sundia Inc. (Shijiazhuang, China) and further purified in the Department of Chemistry of the University of Washington. The drug formulation was prepared by dissolving the compound in 70% Tween 80 (Sigma-Aldrich, Madrid, Spain) and 30% Ethanol 96° (Panreac, Barcelona, Spain) by heating at 60 °C and shaking for 3 h at a final concentration of 69 mg/mL. Starting at 48 h post-infection, BKI-1553 was administered subcutaneously to G1 (1st dose: 35 mg/kg bodyweight, 2nd dose: 10 mg/kg bodyweight a week later) and G3 (10 mg/kg bodyweight, 7 doses every other day). G2 and G4, which represented the corresponding non-infected treatment controls, received the same doses as G1 and G3, respectively. Ewes from G1 and G2 groups were dosed in their armpits with 13.12 ± 0.70 mL for the 1st dose and 8.37 ± 0.93 mL for the 2nd dose. Ewes from groups G3 and G4 were dosed in their armpits and inguinal regions with