

Development of a murine vertical transmission model for *Toxoplasma gondii* oocyst infection and studies on the efficacy of bumped kinase inhibitor (BKI)-1294 and the naphthoquinone buparvaquone against congenital toxoplasmosis

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Objectives: Establishment of a mouse model for congenital toxoplasmosis based on oral infection with oocysts from *Toxoplasma gondii* ME49 and its application for investigating chemotherapeutic options against congenital toxoplasmosis.

Methods: CD1 mice were mated, orally infected with 5, 25, 100, 500 or 2000 oocysts and monitored for clinical signs and survival of dams and pups until 4 weeks *post partum*. The parasite burden in infected mice was quantified by real-time PCR in lungs, brains and, in the case of surviving pups, also in eyes. Seroconversion was assessed by ELISA. *T. gondii* cysts in brain were identified by immunofluorescence. In a second experiment, pregnant CD1 mice challenged with 20 oocysts/mouse were treated with buparvaquone or the calcium-dependent protein kinase 1 inhibitor bumped kinase inhibitor (BKI)-1294 and the outcome of infection was analysed.

Results: *T. gondii* DNA was detected in the brain of all infected animals, irrespective of the infection dose. Seroconversion occurred at 3 weeks post-infection. Most pups born to infected dams died within 1 week *post partum*, but a small fraction survived until the end of the experiment. *T. gondii* DNA was detected in the brain of all survivors and half of them exhibited ocular infection. Chemotherapy with both compounds led to dramatically increased numbers of surviving pups and reduced cerebral infection. Most efficient were treatments with BKI-1294, with 100% survivors and only 7% brain-positive pups.

Conclusions: BKI-1294 and buparvaquone exert excellent activities against transplacental transmission in pregnant mice.

Introduction

Toxoplasma gondii is one of the most widespread parasites worldwide.¹ In most cases, acute infection is asymptomatic. Chronic infection is mostly lifelong, largely persists within the CNS and has long been considered to have no consequences in otherwise healthy individuals.² However, recent studies suggest that chronic infection may induce distinct behavioural changes^{3,4} and may be associated with psychiatric disorders.⁵ *T. gondii* infection is life threatening in immunocompromised persons and may cause

abortion or severe fetal damage when contracted by seronegative pregnant women.^{1,6}

The current treatments of toxoplasmosis are based on a combination therapy comprising sulphonamides and pyrimethamine or other antimicrobials,⁷ including inhibitors of apicoplast division such as doxycycline and minocycline.⁸ There is no approved treatment for maternal and fetal *T. gondii* infections in the USA and many other countries. Therefore, current therapies are not optimal and novel treatment options are required.⁹

A plethora of compounds has been tested against toxoplasmosis¹⁰ and various molecular targets for these compounds have been identified.^{11,12} *T. gondii* is highly amenable to molecular genetics¹³ and has become the prime model organism for apicomplexan parasites. Many of the *in vivo* studies aiming at novel therapeutic options have been performed using either inoculation of tachyzoites or oral feeding of tissue cysts containing bradyzoites of a variety of highly virulent or less virulent strains of *T. gondii*.¹⁴ A comparatively small number of studies on vertical transmission have been conducted using oocysts.¹⁵ Whereas vertical transmission models based on oocyst ingestion are well established in pigs^{16,17} and sheep,^{18–21} small laboratory animal models have not been widely used for this purpose.¹⁸ Mice infected with *T. gondii* M1 strain oocysts prior to gestation transmitted the parasite transplacentally to their offspring¹⁹ and mice that were orally infected with oocysts of two South American *T. gondii* strains showed that vertical transmission occurred with combinations of strains and mouse lines causing negligible clinical signs and mortality of the dams.²⁰

Here we report on a vertical transmission model using outbred CD-1 mice orally infected with oocysts of the *T. gondii* strain ME49 during pregnancy. Furthermore, we demonstrate the suitability of this model for the assessment of therapeutics against congenital toxoplasmosis employing the naphthoquinone buparvaquone and the calcium-dependent protein kinase 1 inhibitor bumped kinase inhibitor (BKI)-1294. Originally developed as an antimalarial,²¹ buparvaquone is active against apicomplexans including *Babesia*,²² *Eimeria* and *Theileria*²³ and against *Leishmania donovani*²⁴ and *Pneumocystis carinii*.²⁵ Buparvaquone inhibits the ubiquinone reductase cytochrome bc.^{25,26} We have shown that buparvaquone is active against *Neospora caninum*,^{27,28} closely related to *T. gondii*. BKI-1294 is effective against cryptosporidiosis in immunosuppressed mice,²⁹ against acute murine toxoplasmosis^{30,31} and against cerebral *N. caninum* infection,³² and interferes with the vertical transmission of neosporosis to newborn pups in a pregnant mouse model.³³

Materials and methods

Biochemicals and drugs

If not stated otherwise, biochemical reagents were from Sigma (St Louis, MO, USA). Buparvaquone was provided by Cross Vetpharm Group Limited (Dublin, Ireland). Chemical synthesis of BKI-1294 was previously described.³⁴

Animal experimentation

All protocols involving animals were approved by the Animal Welfare Committee of the Canton of Bern under the license BE115/14. All animals used in this study were handled in strict accordance with practices designed to minimize suffering. Female and male CD1 mice, 8 weeks of age, were purchased from Charles River (Sulzberg, Germany) and were maintained in a common room according to the guidelines set up by the animal welfare legislation of the Swiss Veterinary Office.

Generation, purification and sporulation of *T. gondii* ME49 oocysts

Sporulated oocysts of *T. gondii* were obtained by oral infection of cats according to methods described elsewhere.³⁵ Briefly, 10 female Swiss Webster mice (Janvier-Labs, Laval, France) aged 8 weeks were inoculated

intraperitoneally with 500 tachyzoites of the ME49 isolate of *T. gondii* suspended in 200 µL of PBS. All infected mice were treated with sulfadimidine sodium (0.3 mg/mL in drinking water) for 8 days, beginning 10 days post-infection, to minimize morbidity and prevent death. At 3 months post-inoculation, mice were humanely euthanized by CO₂ asphyxiation and the brain was removed from each mouse. Two 12-week-old specific-pathogen-free kittens (Isoquimen S.L., Barcelona, Spain) were fed a pool of five brains each from the infected mice. Faeces were collected from kittens daily and examined by saturated sodium chloride solution double centrifugation to detect shedding of *T. gondii* oocysts as well as to monitor possible coinfection with other parasites. Unsporulated oocysts were harvested from faeces using a saturated sodium chloride solution to concentrate them by flotation.³⁶ Oocysts were counted on a haemocytometer and sporulated by re-suspending in 2% H₂SO₄ for 4 days at room temperature. Sporulated oocysts were stored in water at 4°C until further use. All procedures were conducted in a biohazard hood and were approved by the Animal Welfare Committee of Comunidad de Madrid under the license PROEX166/14.

Experiment 1: establishment of the oocyst infection model

Sixty female CD1 mice at 9 weeks of age were synchronized with respect to oestrus as described previously.³⁷ Mice were distributed into cages where two female mice and one male mouse were housed together for 3 days. Subsequently the female mice, randomly allocated to different experimental groups of 10 animals each, were orally infected by gavage with 5, 25, 100, 500 or 2000 purified *T. gondii* ME49 oocysts suspended in 100 µL of carboxymethyl cellulose solution (0.5% in water) at day 7 post-mating. The control group received carboxymethyl cellulose solution alone. Pregnancy was confirmed 2 weeks post-mating by weighing and pregnant mice were then allocated into single cages to give birth on days 19–21 and to rear their pups for an additional 4 weeks. During this time, those females that had remained non-pregnant were maintained in cages of three to five mice. All animals were evaluated daily for clinical signs of disease and were euthanized in a CO₂ chamber when indicated by the attending veterinarian as necessary. Pups were counted each day. Surviving dams and pups were euthanized at 28 days *post partum*, thus 6 weeks post-infection. In the case of the dams and non-pregnant females, blood was recovered by cardiac puncture and sera were obtained to assess humoral immune responses. Brains and lungs were removed for subsequent determination of parasite load and stored at –20°C until further processing. The heads of pups that survived were severed and stored at –20°C. Eyes were removed from the frozen heads using single-use scalpels with narrow tips (No. 11, Lance Paragon, Sheffield, UK). Subsequently, the frozen heads were cleaved and brains were removed. The frozen tissue was immediately processed for DNA purification.

Experiment 2: buparvaquone and BKI-1294 treatments against oocyst-acquired toxoplasmosis in mice

Twenty-four female and 11 male CD1 mice, 10 weeks old, were mated as indicated above and females were randomly allocated to four experimental groups. On day 7 post-mating, they were orally infected with 20 *T. gondii* oocysts. Drug treatments were initiated 2 days post-infection. Infection and beginning of the treatment were scheduled based on our experience with the *N. caninum* mouse model for vertical transmission.^{28,33,37} Buparvaquone and BKI-1294 (each with eight mice per group) were orally applied for 5 days at 50 mg/kg/day; both drugs were emulsified in corn oil. Prior to gavage, the corn oil/drug mixtures were heated to 37°C to enhance solubility of the drug. The placebo group received corn oil alone.

Analysis of biological samples from *in vivo* experiments

To quantify the parasite load in brain and lung tissues, DNA purification was performed employing the NucleoSpin Tissue Kit (Macherey-Nagel, Düren,

Germany) according to the standard protocol suitable for animal tissues. The DNA concentrations in all samples were determined using the QuantiFluor dsDNA System (Promega, Madison, WI, USA) according to the manufacturer's instructions and adjusted with sterile DNase-free water to 5 ng/μL. Quantification of parasite loads in brains and lungs was performed by quantitative real-time PCR as described.^{38,39} Serum titres for *T. gondii* were assessed by ELISA as described.^{40,41}

Detection of *T. gondii* tissue cysts by immunofluorescence

One brain hemisphere each from two mice infected with five *T. gondii* oocysts (Experiment 1) were fixed in 4% paraformaldehyde in PBS overnight and then embedded in paraffin, and sections were placed onto poly-L-lysine-coated glass slides. Specimens were deparaffinized in xylol and rehydrated in a graded series of methanol, blocked in PBS/3% BSA/50 mM glycine (blocking buffer) for 2 h. Subsequently, sections were sequentially incubated for 30 min each in polyclonal anti-TgBAG1 antiserum (1:200 in PBS 0.3% BSA),⁴² anti-rabbit conjugated to FITC (1:400, Sigma-Aldrich), a polyclonal anti-*T. gondii* antiserum diluted 1:2000³³ and an anti-rabbit tetramethylrhodamine (TRITC) conjugate (1:400, Sigma-Aldrich). After antibody incubations, sections were washed in PBS for 5 min. Sections were embedded in Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). All specimens were viewed on a Nikon Eclipse E800 digital confocal fluorescence microscope. Processing of images was performed using Openlab 5.5.2 software (Improvision, PerkinElmer, Waltham, MA, USA).

Statistical methods

Statistical analysis of the parasite burdens in brains was done using the Kruskal–Wallis test followed by the Wilcoxon rank-sum test with Holm adjustment. Nominal data were analysed using the χ^2 test for independence and considering *P* values <0.01 as statistically significant. All analyses were performed using the software package R.⁴³

Results

Set-up of an oocyst infection model in CD1 mice

To determine a degree of infection suitable for subsequent experiments, female CD1 mice were mated and orally infected with a concentration series of *T. gondii* ME49 oocysts, or remained uninfected, as detailed in the Materials and methods section. The mice were monitored for clinical signs daily for a maximum of 6 weeks. Mice exhibiting clinical signs early after infection were euthanized at the onset of disease manifestation, those mice that did not get pregnant or that gave birth to pups suffering from neonatal mortality were sacrificed at 3 weeks post-infection (1 week *post partum*) and asymptomatic dams with pups were euthanized 4 weeks *post partum*, thus 6 weeks post-infection. Thus, the three endpoints analysed were: (i) prior to 3 weeks post-infection; (ii) 3 weeks post-infection; and (iii) 6 weeks post-infection. Samples from lung and brain tissues were assessed by quantitative RT-PCR and sera were analysed by ELISA. The numerical results are summarized in Table 1.

In the group that received 2000 oocysts, 9 of the 10 mice died prior to 3 weeks post-infection (3 animals) or they had to be euthanized due to the severity of clinical signs (6 animals). In all nine mice *T. gondii* DNA was detected in the brain and in eight animals also in the lungs. Blood samples could be drawn only from the six animals that had to be euthanized and none of these was

Toxoplasma ELISA positive. One mouse from this group survived until the end of the experiment and had seroconverted. None of the mice of this group was pregnant, while in the non-infected control group 7 out of 10 mice gave birth.

The pregnancy rate was higher in the groups with lower inoculation doses: 4 out of 10 in mice infected with 500 oocysts and 8 out of 10 in mice receiving 5 oocysts. Cerebral and lung infection was detected in all infected animals that were analysed. All animals sacrificed at 3 weeks post-infection or later had mounted an antibody response against *T. gondii* (Table 1). Quantitative evaluation of parasite burdens and serum titres as a function of the time of death showed a clear increase in both parameters between early and late endpoints (Figure 1a and b).

The litter size was markedly decreased in the group of (surviving) dams that received 500 oocysts, whereas in the non-infected control group all pups survived. There was a pronounced mortality in all groups that received oocysts, with only 22 surviving pups in the groups infected with 5, 25 and 100 oocysts. All brains of surviving pups tested *Toxoplasma* positive by PCR. In 8 of these 22 pups, *T. gondii* could also be detected in the eyes (Table 1).

The survival curve of the pups showed a marked decrease in survivors within the first week *post partum* in all groups, followed by a low but stable level until the end of the experiment. Whenever possible, dead pups succumbing to the infection early after birth were removed, their heads were sampled and their brains analysed as well. It was not possible to recover all dead pups, but the brains of 14 that had died during the first week after birth could be analysed. Four of five pups that had died at day 5 *post partum* or later were brain positive when analysed by *T. gondii* PCR. No *T. gondii* DNA could be detected in the brain of pups that had died prior to 5 days *post partum*.

Tissue cyst formation after oral infection with oocysts

In order to visualize tissue cyst formation, one brain hemisphere from two dams each inoculated with five oocysts and sacrificed after 6 weeks post-infection (Table 1) was processed by immunohistology. As shown in Figure 2, tissue cysts could be identified by staining brain slices with antiserum raised against *T. gondii*-BAG1. The staining was specifically located in the cytoplasm of the bradyzoites, while the antiserum directed against whole *Toxoplasma* extract additionally labelled the cyst wall (Figure 2). Typically, two to four tissue cysts could be found per section.

Treatments of pregnant mice with BKI-1294 and buparvaquone protect dams and pups from vertical transmission

The oocyst infection model was applied to assess the effects of buparvaquone and the calcium-dependent protein kinase 1 inhibitor BKI-1294 as described. Based on the results presented above, we selected an inoculation dose of 20 oocysts to ensure a good infection without risking premature death. All dams from the placebo and buparvaquone-treated groups, but only four out of eight from the BKI-1294 group, gave birth to offspring (Table 2). All dams survived at least until 3 weeks post-infection (thus until 1 week *post partum*). At this timepoint, four dams from the placebo group and one dam from the buparvaquone-treated group with clinical signs and with dead pups, the four non-pregnant

Table 1. Effects of oral infection with *T. gondii* ME49 oocysts on mortality, fertility and infection status in non-pregnant mice, dams and pups

Oocysts	endpoint	Dams					Pups			
		<i>n</i>	lung positive	brain positive	serum positive	pregnant	litter size (0 days <i>post partum</i>)	surviving (28 days <i>post partum</i>)	brain positive (28 days <i>post partum</i>)	eye positive (28 days <i>post partum</i>)
5	<3 wpi	1	1/1 ^a	1/1	1/1	8/10	52	10 (19%) ^{b***}	10 (100%) ^c	3 (33%)
	3 wpi	5	4/5	5/5	5/5					
	6 wpi	4	ND	4/4	4/4					
25	<3 wpi	3	3/3	3/3	1/3	6/10	39	3 (8%) ^{***}	3 (100%)	2 (66%)
	3 wpi	1	1/1	1/1	1/1					
	6 wpi	6	ND	6/6	6/6					
100	<3 wpi	3	3/3	3/3	3/3	5/10	36	9 (25%) ^{***}	9 (100%)	3 (33%)
	3 wpi	1	1/1	1/1	1/1					
	6 wpi	6	ND	6/6	6/6					
500	<3 wpi	4	4/4	4/4	0/2	4/10	10*	0 (0%) ^{***}	-	-
	3 wpi	1	1/1	1/1	1/1					
	6 wpi	5	ND	5/5	5/5					
2000	<3 wpi	9	8/9	9/9	0/6	0/10**	-	-	-	-
	6 wpi	1	ND	1/1	1/1					
Control						7/10	83	83		

ND, not detected; wpi, weeks post-infection.

Female CD1 mice were mated, inoculated intragastrically with various amounts of purified *T. gondii* ME49 oocysts or left uninfected (control), and euthanized as described in the Materials and methods section. Adults were tested for *T. gondii* seropositivity. Adults and surviving pups were tested for the presence of *T. gondii* in their brains and lungs (dams only) by real-time PCR. Fertility data of animals in infected and control groups were compared by χ^2 tests (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$).

^aRead 1 of 1.

^bPercentage of initial number of pups.

^cPercentage of number of surviving pups.

females from the BKI-1294 group and the three non-pregnant females from the combined group were sacrificed. The other dams and the surviving pups were sacrificed 4 weeks *post partum*, thus 6 weeks post-infection.

At 6 weeks post-infection, the four surviving dams from the placebo group also showed clinical signs. The dams from the treated groups were all free of clinical signs. All dams were seropositive and PCR positive in their brain (except two dams from the BKI-1294 group) and PCR positive in the lungs (Table 2).

The parasite burdens in brains and lungs of dams sacrificed after 3 and 6 weeks overlapped (Figure 3a and b) and therefore were analysed together. Comparison of the brain values revealed a statistically significant difference (Kruskal-Wallis test; $P = 0.015$); multiple comparison revealed that the cerebral parasite loads of the group treated with BKI-1294 were significantly different from those of the placebo group (Wilcoxon test; $P = 0.03$). The lung values were not significantly different, but more than one order of magnitude smaller than the brain values (Figure 3a and b).

All three treatment schemes provided strong protection of the pups from congenitally transmitted toxoplasmosis. With the exception of five pups in the buparvaquone-treated group that died within the first week, all pups from the treatment groups survived, whereas 55 out of 80 pups from the placebo group died within the first week (Figure 3c). This effect on survival was highly significant, as shown by χ^2 tests of the numbers of surviving pups (Table 2).

Only 12 of the surviving pups were, however, brain positive. Nevertheless, the ratios between positive and negative pups in the treated groups were still significantly different from those in the placebo group when only surviving pups were considered. The treatment effect is even much more pronounced if we consider the pups that died prior to 4 weeks *post partum* also as *T. gondii* positive (Table 2).

Discussion

To develop new chemotherapies against congenital toxoplasmosis, we have established a mouse model based on a challenge of dams by oral infection with *T. gondii* ME49 oocysts. This model is based on previous achievements using a standardized vertical transmission model of neosporosis.^{27,33,37,44}

In this study, oocyst-infected dams showed severe clinical signs even with a dose as low as five oocysts per animal (equalling not more than 40 potentially invasive sporozoites). The cerebral parasite burden increased with time and resulted in the formation of clearly detectable tissue cysts. Vertical transmission was highly efficient and resulted in high mortality within the first week *post partum*. However, not all dead pups collected during the first 3 weeks after birth were *T. gondii* positive in their brain. Several reasons could account for this: (i) their parasite numbers were below the detection limit; (ii) they had died of multiple organ failure and not

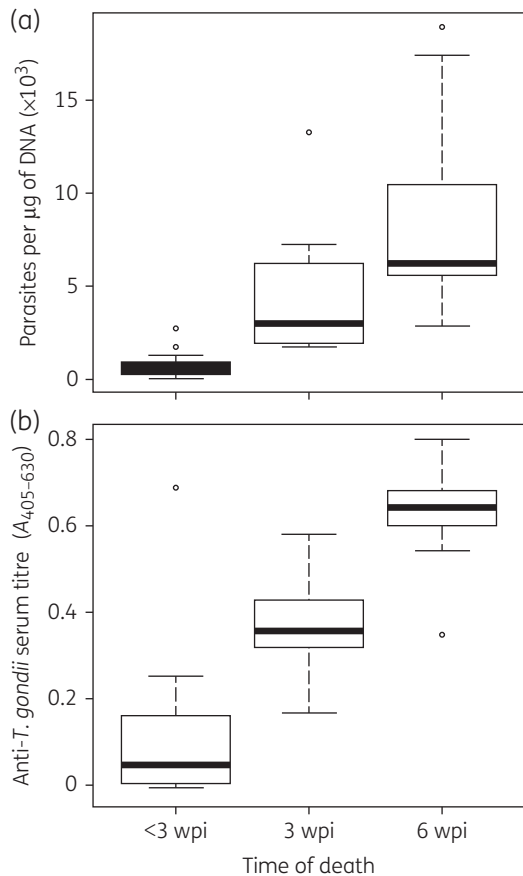


Figure 1. Infection status of dams and survival of pups after oral inoculation of dams with *T. gondii* ME49 oocysts. CD1 mice were mated and inoculated with *T. gondii* ME49 oocysts, and infection parameters were analysed as described in the Materials and methods section and in Table 1. Brain parasite burdens quantified by RT-PCR (a) and anti-*T. gondii*-serum titres quantified by ELISA (b) are presented according to the time of death of the dams. wpi, weeks post-infection.

due to cerebral infection; or (iii) death was not caused by direct infection of the fetus, but rather due to physiological dysregulation and/or extensive damage within the placental tissue. Subsequently to 1 week *post partum*, the numbers of survivors remained stable. This infection status can be regarded as chronic, thereby simulating the situation in human newborns after transplacental infection. Since ocular toxoplasmosis is a major issue in newborns,⁶ we determined whether ocular or cerebral infection gives a more predictive parameter for the establishment of chronic toxoplasmosis. *T. gondii* was detected in 8 of the 22 surviving pups.

We suggest that this oocyst model could be used to assess the effects of novel chemotherapeutics against *T. gondii* infection. The advantages of this model reside: (i) in a more natural way of infection of dams and offspring; (ii) in the establishment of congenital toxoplasmosis including ocular infection; and (iii) in the possibility of long-term studies of treatment efficacies. A disadvantage of this model is the long-term use of experimental animals and, consequently, the need for qualified personnel. Moreover, the variation in such experiments is inherently greater than in short-term *in vivo* or *in vitro* experiments. In addition, one has to keep in mind that

oocysts, although stable over a period of several months when kept at 4°C, will evidently lose infectivity with time, hence this could be a reason for the variability in the number of pregnant dams seen between Experiment 1 (6 out of 10 when infected with 25 oocysts) and Experiment 2 (carried out 3 months later, 8 of 8 pregnant and infected with 20 oocysts). Most cases of congenital toxoplasmosis in Europe are caused by type II strains, such as ME49, but type I or atypical strains causing clinical signs have also been isolated. One of the important parameters that determine whether fetal infection occurs is the time during gestation at which maternal challenge takes place. Based on our previously established neosporosis model,³⁷ we have here chosen day 7 post-mating, which represents the beginning of the second trimester of gestation in mice. In humans, most cases that are born with clinical signs are infected during the second trimester of gestation. Infection prior to this date often causes abortion and infection in the third trimester leads less frequently to fetal harm.¹⁰ However, to what extent our model compares with the situation in humans or other animal species needs further clarification.

Buparvaquone and BKI-1294 have been successfully tested against vertically transmitted neosporosis in previous experiments.^{28,33} Buparvaquone rapidly impairs the viability of the apicomplexan parasite *Theileria*.^{26,45} In this experiment, pups of buparvaquone-treated dams exhibited significantly higher survival compared with the placebo pups; however, the cerebral parasite burden in the dams was similar to that in the placebo group. The time window between treatment and birth was apparently sufficient to ensure protection from transmission. Besides the fact that buparvaquone does not cross the blood-brain barrier, the solubility of the drug is poor and maximum plasma concentrations are in the sub-micromolar range.⁴⁶ Novel prodrug formulations may, however, overcome this handicap.⁴⁷⁻⁴⁹

Dams treated with BKI-1294 had significantly lower parasite burdens, remained free of clinical signs and all pups survived until the end of the experiment at 4 weeks *post partum*. Only 50% of the dams were, however, pregnant, suggesting a detrimental effect of BKI-1294 on fertility. This effect was not visible in previous experiments with BKI-1294 using BALB/c mice, either for physiological reasons or due to the fact that the overall pregnancy rate is lower in inbred BALB/c mice compared with the outbred CD1 mice.³³ However, more recent studies have demonstrated marked effects of other BKIs on fertility in BALB/c mice.⁵⁰ The reason for these effects remains to be elucidated. The treatment with BKI-1294 was sufficient to abolish clinical signs in all offspring and resulted in only 4 of 55 (7%) pups with detectable *T. gondii* DNA in their brain. In comparison, in the buparvaquone treatment group 17 of 88 (19%) of the offspring were infected. How BKI-1294 exerts this excellent efficacy is not clear. In both *N. caninum* and *T. gondii* the compound was not parasitocidal, but caused the transformation of intracellular tachyzoites into multinucleated complexes, which exhibited deregulated SAG1 and BAG1 antigen expression.³³ The prolonged effect of BKI-1294 cannot be explained by its action merely during the 5 days of treatment. Possibly, BKI-1294 treatment transformed *T. gondii* into a non-virulent status, thus acting in a manner similar to an attenuated live vaccine. Clearly, this aspect deserves further attention. Previous studies have shown that infection with a non-virulent *N. caninum* strain protected mice against a lethal challenge by a virulent *T. gondii* strain.⁵¹ Future

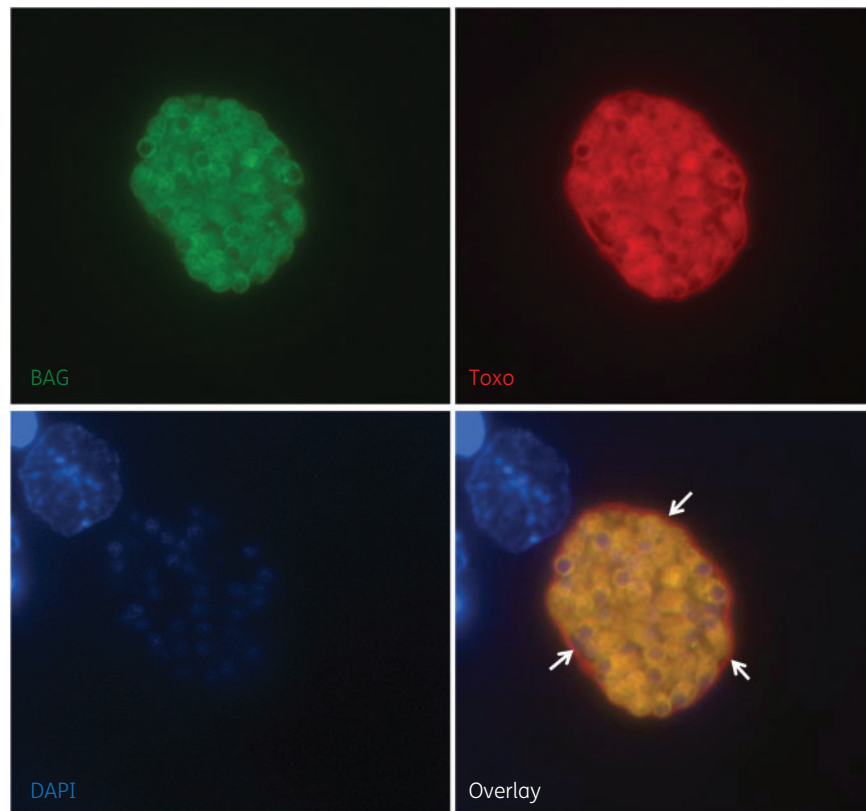


Figure 2. *T. gondii* ME49 forms tissue cysts after oral oocyst inoculation. Brain pieces of dams inoculated with five oocysts and euthanized after 6 weeks post-infection (see Table 1 and Figure 1) were processed for immunofluorescence performed with anti-*T. gondii*-BAG1 (BAG, green) and anti-*T. gondii* antiserum (Toxo, red) as described in the Materials and methods section. The blue dye DAPI indicates host cell and parasite nuclei. Arrows in the overlay point to the cyst wall stained with anti-*T. gondii* antiserum, which is not labelled by BAG1 antibody. This figure appears in colour in the on-line version of JAC and in black and white in the print version of JAC.

Table 2. Effects of treatments with buparvaquone and BKI-1294 alone or in combination on fertility and parasite burden in dams and pups after oral infection of dams with *T. gondii* ME49 oocysts

Group	Dams				Pups			
	pregnant	brain positive	lung positive	serum positive	litter size (0 days post partum)	surviving (28 days post partum)	brain positive (28 days post partum)	<i>T. gondii</i> positive (total)
Placebo	8/8 (100%)	8/8	8/8	8/8	80 (100%)	25 (31%)	12 (48%) ^a	67 (84%) ^b
Buparvaquone	8/8	8/8	8/8	8/8	99 (100%)	88 (89%)***	17 (19%)*	22 (22%)***
BKI-1294	4/8 (50%)	6/8 (75%)	8/8	8/8	55 (100%)	55 (100%)***	4 (7%)***	4 (7%)***
χ^2 (df = 2)	6.93	3.03	ND	ND		85.3	18.1	101.6
P	0.03	0.22				3.0×10^{-19}	1.2×10^{-4}	8.7×10^{-23}

Female CD1 mice were mated, inoculated with *T. gondii* oocysts, treated with buparvaquone (suspended in corn oil), BKI-1294 (suspended in corn oil) or corn oil alone (placebo) and euthanized as described in the Materials and methods section. Adults and surviving pups were tested for the presence of *T. gondii* in their brains by real-time PCR. Data were analysed by multiple χ^2 tests and respective numbers of animals in treated and placebo groups were compared by single χ^2 tests (* $P < 0.01$, *** $P < 0.0001$).

^aPercentage of survivors.
^bPercentage of total pups.

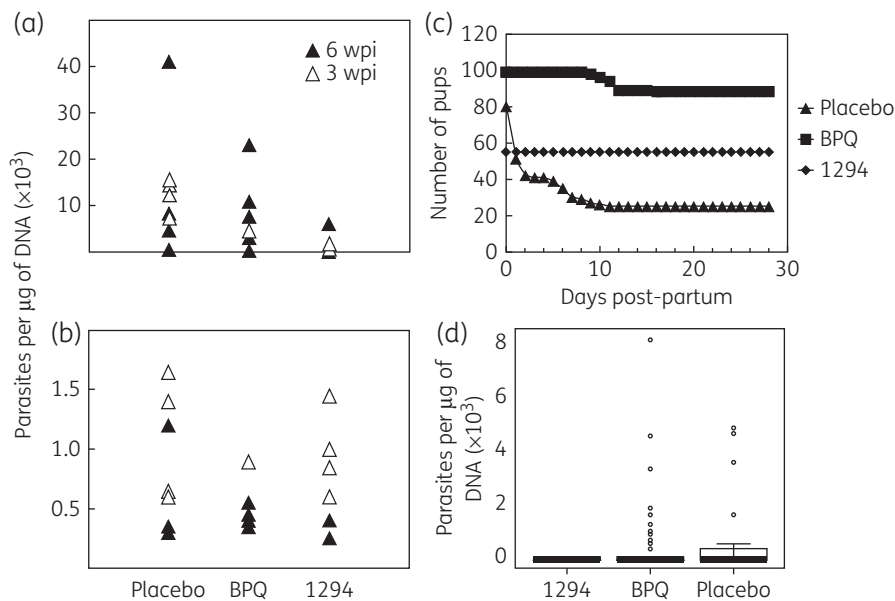


Figure 3. Quantitative assessments of parasite burdens in dams and pups and survival of pups in the oocyst infection model as a consequence of treatments with buparvaquone or BKI-1294. Female CD1 mice were mated, inoculated with *T. gondii* ME49 oocysts, subsequently treated with buparvaquone or BKI-1294 and euthanized as described in the Materials and methods section and in Table 2. (a) and (b) Parasite burden in dams. After euthanasia of dams, brains and lungs were collected and the parasite burdens as determined by quantitative PCR are presented as open symbols (euthanasia at 3 weeks post-infection) or filled symbols (euthanasia at 6 weeks post-infection) for each dam. Values in brains are shown in (a) and values in lungs are shown in (b). Survival curves of pups are shown in (c), and (d) shows the cerebral parasite burdens of surviving pups euthanized 28 days *post partum*. 1294, BKI-1294; BPQ, buparvaquone; wpi, weeks post-infection.

studies with our model will test this hypothesis with even less virulent strains than ME49, thus allowing repeated challenges.

In conclusion, we have established a mouse model to test chemotherapeutic or other therapy options against congenital toxoplasmosis caused by ingestion of oocysts, and demonstrated protection in a study with two promising candidate compounds that should be followed up in future studies.

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Transparency declarations

W. V. V. is the founder of the company ParaTheraTech Inc., dedicated to bring BKIs to market in animal health indications. All other authors: none to declare.

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