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In Vitro and *In Vivo* Effects of the Bumped Kinase Inhibitor 1294 in the Related Cyst-Forming Apicomplexans *Toxoplasma gondii* and *Neospora caninum*

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We report on the *in vitro* effects of the bumped kinase inhibitor 1294 (BKI-1294) in cultures of virulent *Neospora caninum* isolates Nc-Liverpool (Nc-Liv) and Nc-Spain7 and in two strains of *Toxoplasma gondii* (RH and ME49), all grown in human foreskin fibroblasts. In these parasites, BKI-1294 acted with 50% inhibitory concentrations (IC_{50}s) ranging from 20 nM (*T. gondii* RH) to 360 nM (*N. caninum* Nc-Liv), and exposure of intracellular stages to 1294 led to the nondisjunction of newly formed tachyzoites, resulting in the formation of multinucleated complexes similar to complexes previously observed in BKI-1294-treated *N. caninum* beta-galactosidase-expressing parasites. However, such complexes were not seen in a transgenic *T. gondii* strain that expressed CDPK1 harboring a mutation (G to M) in the gatekeeper residue. In *T. gondii* ME49 and *N. caninum* Nc-Liv, exposure of cultures to BKI-1294 resulted in the elevated expression of mRNA coding for the bradyzoite marker BAG1. Unlike in bradyzoites, SAG1 expression was not repressed. Immunofluorescence also showed that these multinucleated complexes expressed SAG1 and BAG1 and the monoclonal antibody CC2, which binds to a yet unidentified bradyzoite antigen, also exhibited increased labeling. In a pregnant mouse model, BKI-1294 efficiently inhibited vertical transmission in BALB/c mice experimentally infected with one of the two virulent isolates Nc-Liv or Nc-Spain7, demonstrating proof of concept that this compound protected offspring from vertical transmission and disease. The observed deregulated antigen expression effect may enhance the immune response during BKI-1294 therapy and will be the subject of future studies.

Neospora caninum is a cyst-forming apicomplexan parasite that is closely related to *Toxoplasma gondii* but exhibits distinct differences in transmission patterns, virulence, host specificity, immunogenetic aspects, and the pathology it induces. *T. gondii* causes toxoplasmosis in humans and many domestic and wildlife animals, with great economic impact especially in sheep but also in many other animal species (1). Human toxoplasmosis causes serious pathology in immune-suppressed individuals. In addition, if a seronegative mother acquires primary infection during pregnancy, human toxoplasmosis can lead to abortion, microcephalus and hydrocephalus, and other fetal abnormalities causing intellectual disability (2). *N. caninum* is a veterinary health problem and represents one of the most important infectious causes of bovine abortion, stillbirth, and the birth of weak calves, with an economic impact of over $1.3 billion (3–5). In addition, *N. caninum* causes neuromuscular disease in dogs, and neosporosis has also been detected in a wide range of other species of livestock and wild animals worldwide.

Despite their differences, an important common feature of these parasites is their ability to invade and replicate within a wide range of cell types and tissues, where they reside in an intracellular parasitophorous vacuole, surrounded by a parasitophorous vacuole membrane. Repeated cycles of invasion, proliferation, and egress of the disease-causing tachyzoites are responsible for inducing the pathological effects that occur during the acute stage of infection. In turn, chronic infection is characterized by the formation of intracellular tissue cysts in brain and muscular tissues that harbor slowly proliferating bradyzoites. Calcium-dependent protein kinases (CDPKs), encoded by apicoplast-associated genes and, thus, only found in apicomplexan parasites and plants, represent excellent drug targets in several apicomplexans such as *Plasmodium falciparum* (6), *Cryptosporidium parvum* (7, 8) where novel drug targets are of crucial interest (9), *N. caninum* (10), *Eimeria tenella*, and *Babesia bovis* (11). Excellent correlations between cell activity and CDPK1 inhibition were achieved by compounds from a focused bumped kinase inhibitor (BKI) library. In many apicomplexan CDPK1 enzymes, including *T. gondii* and *N. caninum*, the ATP binding pocket is characterized by a glycine as the smallest possible gatekeeper residue. The *T. gondii* strain CDPK1_G128M, overexpressing a CDPK1 version with a muta-

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tion (G to M) in the ATP binding pocket, was found to be much less sensitive to BKIs (12).

We have previously demonstrated the outstanding efficacy of BKI-1294 against transgenic beta-galactosidase-expressing *N. caninum* tachyzoites (Nc-betaGal) *in vitro* and in a nonpregnant mouse model for cerebral infection (10). In addition, we have shown that for the BKI-1294-inhibited egress of Nc-betaGal tachyzoites *in vitro*, once located intracellularly, parasites underwent nuclear division but cytokinesis was not achieved, which resulted in the formation of large multinucleated complexes that remained trapped within the host cell and eventually died (10). However, it is currently unclear whether this incomplete cytokinesis was caused specifically by CDPK1 inhibition or whether another target may be potentially involved (13).

In this study, we compared *in vitro* effects of BKI-1294 in tachyzoite cultures of the virulent *N. caninum* isolates Nc-Liverpool (Nc-Liv) and Nc-Spain7 and in two strains of *T. gondii* (RH and ME49). In order to investigate the direct role of CDPKI, we have included the *T. gondii* strain overexpressing the gatekeeper mutation G128M and a control strain overexpressing the wild-type CDPKI in our study. We show that BKI-1294 does not only interfere in tachyzoite invasion but also causes incomplete cytokinesis resulting in the formation of multinucleated complexes in the two species but not in the transgenic *T. gondii* strain expressing CDPK1 harboring a mutation (G to M) in the gatekeeper residue. Moreover, we present data on antigen expression in these multinucleate complexes. We also show that BKI-1294 inhibits vertical transmission of Nc-Liv and Nc-Spain7 in a pregnant mouse model for *N. caninum* infection. Our data provide a proof of concept for the treatment of *N. caninum* infection and the protection of offspring by BKI-1294 and related compounds.

MATERIALS AND METHODS

Tissue culture media, biochemicals, and drugs. If not stated otherwise, all tissue culture media were purchased from Gibco-BRL (Zürich, Switzerland), and biochemical reagents were from Sigma (St. Louis, MO). Kits for molecular biology were purchased from Qiagen (Hilden, Germany). BKI-1294 was obtained from the Center for Emerging and Reemerging Infectious Diseases (CERID), Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington (Seattle, WA, USA). For *in vitro* studies, the compound was stored as a 20 mM stock solution in dimethyl sulfoxide (DMSO) at −20°C; for application in mice, BKI-1294 powder was suspended either in 0.5% carboxymethyl cellulose (CMC) in water or in corn oil, and the suspensions were applied by gavage.

Host cell cultivation and parasite cultures. Human foreskin fibroblasts (HFF) were maintained in Dulbecco's modified Eagle medium (DMEM), and Vero cells were cultured in RPMI 1640 medium, each with phenol red supplemented with 10% heat-inactivated and sterile filtered fetal calf serum (FCS), 50 U of penicillin/ml, and 50 μg streptomycin/ml (culture medium). Cultures were maintained at 37°C and 5% CO_2 in tissue culture flasks (Sarstedt, Sevelen, Switzerland) and were passaged at least once a week.

Tachyzoites of *N. caninum* isolates Nc-Liverpool (Nc-Liv) and Nc-Spain7, *T. gondii* strains ME49 and RH, the *T. gondii* strain CDPK1_G128M overexpressing a CDPK1 with the gatekeeper mutation G128M, and an isogenic strain, *T. gondii* CDPK_wt, expressing the wild-type CDPK1 (12) were all maintained by serial passages in either Vero cells or HFF in their respective media (14, 15). Tachyzoites were harvested by removing infected cell layers with a rubber cell scraper followed by repeated passages through a 25-gauge needle at 4°C. For *in vitro* studies, tachyzoites were separated from cell debris on a Sephadex G-25 column (16). For *in vivo* experiments, purification over Sephadex G-25 columns was omitted.

Determination of 50% inhibitory concentrations. HFF were grown in 6-well plates for 24 h until a confluent monolayer was formed. Just prior to infection, the BKI-1294 was added at a final concentration of 2.5 μM for initial experiments and ranged between 0.5 nM and 2.5 μM for 50% effective concentration (EC_{50}) determinations (the effective concentration to reduce proliferation by 50%). Controls received the corresponding amounts of DMSO. HFF were then infected with 5×10^4 freshly purified tachyzoites of *N. caninum* Nc-Liv, Nc-Spain7, *T. gondii* ME49, *T. gondii* CDPK1_G128M, or *T. gondii* CDPK1_wt in 5 ml medium. After 4 days, cells were collected with a cell scraper, centrifuged, washed once more in phosphate-buffered saline (PBS), and the pellet was stored at −20°C prior to quantification of parasite proliferation. DNA purification was performed employing the DNeasy blood and tissue kit (Qiagen, Basel, Switzerland) according to the standard protocol suitable for animal cells. *N. caninum* parasite load was determined by real-time PCR as previously described (15). Proliferation of *T. gondii* ME49, *T. gondii* CDPK1_G128M, and *T. gondii* CDPK1_wt tachyzoites was quantified as described previously (17). The parasite counts were calculated by interpolation from a standard curve with DNA equivalents from 1,000, 100, and 10 culture-derived tachyzoites included in each run.

Transmission electron microscopy. HFF were grown to confluence in T25 flasks and were infected with *T. gondii* ME49, *T. gondii* RH, *T. gondii* CDPK1_G128M, *T. gondii* CDPK1_wt, or *N. caninum* Nc-Liv tachyzoites. After 24 h, cultures were supplemented with 2.5 μM BKI-1294; infected cultures treated with DMSO served as controls. After 2, 4, and 6 days of treatment, the monolayers were washed with 100 mM sodium cacodylate buffer (pH 7.3) and fixed with cacodylate buffer containing 2.5% glutaraldehyde for 10 min (18). Cells were collected using a rubber cell scraper and centrifuged for 10 min at 1,200 rpm and room temperature. The supernatant was removed, and infected cells were fixed further in glutaraldehyde-cacodylate at 4°C overnight. Postfixation in 2% OsO_4, dehydration, embedding in Epon 820 epoxy resin, and cutting of ultrathin sections was done as previously described (18, 19). Specimens were viewed on a Phillips 400 transmission electron microscope (TEM) operating at 80 kV.

Measurement of BAG1 and SAG1 RNA expression during drug treatment by reverse transcriptase real-time PCR. Confluent HFF monolayers grown in 6-well plates were infected with 2.5×10^4 *N. caninum* or *T. gondii* tachyzoites. Treatments with 5 μM BKI-1294 or DMSO alone were initiated 2 days postinfection (p.i.). After different time points, medium supernatant was removed, the monolayers were washed once in PBS, and the adherent cells were carefully removed with a rubber cell scraper. The suspension was transferred to a tube, and after centrifugation at $600 \times g$ for 10 min at 4°C, the supernatant was removed and the pellet resuspended in 350 μl RLT buffer of the RNeasy minikit (Qiagen, Hilden, Germany) and stored at −20°C until further use. RNA extraction was carried out according to the instructions provided by the manufacturers. Synthesis of cDNA for quantitative reverse transcriptase PCR (RT-PCR) was performed with 2 μg of RNA using the Qiagen Omniscript kit with random primers according to the manufacturer's instructions. Quantitative PCR was performed with 10 μl of cDNA (diluted 1:50 in water) using the QuantiTect SYBR green PCR kit (Qiagen) in 20 μl standard reaction mixtures containing 0.5 μM forward and reverse primers (MWG Biotech, Ebersberg, Germany) as listed in Table 1. Furthermore, control PCRs with RNA equivalents from samples that had not been reverse transcribed into cDNA (data not shown) confirmed that no DNA was amplified from any residual genomic DNA that might have resisted DNase I digestion (see above). PCR was started by initiating the hot-start *Taq* DNA-polymerase reaction at 95°C (10 min). Subsequent DNA amplification was performed in 40 cycles, including denaturation (94°C for 15 s), annealing (58°C for 15 s), and extension (72°C for 30 s); temperature transition rates in all cycle steps were 20°C/s. Fluorescence was measured at 79°C on a Corbett cycler (Corbett Research, Mortlake, Australia). From the quantitative RT-

TABLE 1 Overview of primers used in this study

Primer	Gene product (accession no.[a])	Sequence
NcSAG1-F	*N. caninum* SAG1 (FR823385; region 1666638–1673366)	GGGTCGTAGGCTTGATGG
NcSAG1-R		GCAGCAGCGGCAAGGGG
TgSAG1-F	*T. gondii* SAG1 (TGME49_264660)	CTTCAGGACGTCGTTCCC
TgSAG1-R		CACCTTTGCAGGCTTGCC
NcBAG1_F	*N. caninum* BAG1 (NCLIV_chrVIIb; region 2868187–2869925)	CTCGACTTCATGGATGAGG
NcBAG1_R		CTTCTATGGTAACGTCATCC
TgBAG1-F	*T. gondii* BAG1 (TGME49_259020)	CTAGACTATTTGGAT
TgBAG1-R		CTGTCGAACTCCACG
NcGra2_F	*N. caninum* GRA2 (AF196293.1)	GCCATGCGTGTATCGCCC
NcGra2_R		TTCGCAGTAAACTGCTTGAG
TgGra2_F	*T. gondii* GRA2 (TGME49_227620)	CCAGAAGAACCGGTTTCCC
TgGra2_R		AACGGTCACCATGCCCCT

[a] The accession numbers for *T. gondii* SAG1, BAG1, and GRA2 are from ToxoDB (http://www.toxodb.org), and the accession number for *N. caninum* BAG1 is from GeneDB (http://www.genedb.org).

PCR, mean values (MV) (±standard error [SE]) from four replicates were assessed, and expression levels were given as values in arbitrary units relative to the amount of Gra rRNA.

Immunofluorescence staining. HFF monolayers grown in 24-well plates on poly-L-lysine coated coverslips were infected with 2×10^4 *N. caninum* Nc-Liv or *T. gondii* ME49 tachyzoites. After initial growth for 24 h, specimens were exposed to 2.5 μM BKI-1294 at periods of 2, 4, or 6 days. They were then washed once in PBS and fixed and processed for immunofluorescence as previously described (18). The following antibodies (Abs) were used: (i) a whole *N. caninum* extract rabbit antiserum (16), diluted 1:2,500; (ii) mAbCC2, a rat monoclonal Ab reacting with a yet uncharacterized cyst wall antigen (20), diluted 1:300; (iii) α-BAG1, a polyclonal rabbit Ab directed against BAG1, a classical bradyzoite-specific antigen (21–23), diluted 1:300; (iv) α-SAG1, a mouse monoclonal Ab directed against the major immunodominant tachyzoite surface antigen (24), diluted 1:300; and (v) a whole *T. gondii* extract rabbit antiserum, diluted 1:2,000. Primary antibodies were applied for 45 min at room temperature in PBS with 0.3% bovine serum albumin (BSA). After three washes in PBS for 5 min each, the corresponding secondary antibody conjugates (anti-mouse fluorescein isothiocyanate [FITC] or anti-rabbit Texas Red [Sigma-Aldrich]) were applied at a dilution of 1:300 in PBS with 0.3% BSA. After three washes in PBS for 5 min each, specimens were incubated in 4′,6-diamidino-2-phenylindole (diluted 1:300 in PBS) and then embedded in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). All specimens were viewed on a Nikon Eclipse E800 digital confocal fluorescence microscope. Processing of images was performed using the Openlab 5.5.2 software (Improvision, PerkinElmer, Waltham MA, USA).

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 made to minimize suffering.

Assessment of potential effects of the BKI-1294 on pregnancy outcome in noninfected BALB/c mice. Animal procedures were approved by the Animal Welfare Committee of the Canton of Bern (approval no. BE115/14). Forty female and 20 male BALB/c mice, 8 weeks of age, were purchased from a commercial breeder (Charles River, Sulzberg, Germany) and were maintained in a common room under controlled temperature and a 14-h dark and 10-h light cycle according to the guidelines set up by the animal welfare legislation of the Swiss Veterinary Office (approval no. BE 105/14). Female mice were oestrus-synchronized by the Whitten effect and were mated for three nights by housing 1 male with 2 females (25). At day 9 of pregnancy, the pregnant females were randomly distributed into 2 experimental groups of 10 females each. One group was treated with the BKI-1294 (50 mg/kg of body weight per day emulsified in 100 μl 0.5% CMC in water) for a period of 8 days by gavage once a day, and the other group was treated identically with CMC but without drug. Mice were separated into individual cages at day 18 of pregnancy, and they gave birth on days 20 to 22. Dams and offspring were maintained for at least 1 month after birth and were closely monitored to rule out any adverse effects due to drug treatment.

Assessment of the efficacy of treatment with the BKI-1294 in mice infected with the *N. caninum* Nc-Liv isolate. Thirty female and 15 male BALB/c mice, 8 weeks of age, were maintained as described above. Pregnancy was achieved after synchronization of oestrus followed by mating (1 male housed with 2 females) for 3 nights. They were all infected by intraperitoneal (i.p.) inoculation of 2×10^6 *N. caninum* Nc-Liv tachyzoites on day 7 postmating. Following infection and for the duration of the entire experiment, mice were inspected twice daily for clinical signs (ruffled coat, apathy, and hind limb paralysis) and were categorized according to a score sheet approved by the local authorities. Two days after infection, 12 pregnant and 12 nonpregnant mice were randomly allocated into experimental groups of 6 (2 pregnant and 2 nonpregnant groups). One pregnant and one nonpregnant group were given the BKI-1294 (50 mg/kg/day emulsified in 100 μl 0.5% CMC in water) for a period of 8 days by oral gavage once a day, while the other two control groups received CMC in water. Pregnant mice were placed into individual cages on day 18 of pregnancy, and they all gave birth on days 20 to 22. Dams were euthanized in a CO_2 chamber and offspring decapitated immediately after birth. The nonpregnant mice were euthanized in a CO_2 chamber at 15 days p.i. Brains and sera were sampled and stored at −20°C for subsequent analyses.

Assessment of the efficacy of treatment with the BKI-1294 in nonpregnant and pregnant BALB/c mice experimentally infected with the *N. caninum* Nc-Spain7 isolate. Sixty female and 30 male BALB/c mice were housed, and pregnancy was achieved after synchronization of oestrus as described above. Subsequently, female mice were randomly distributed in 3 groups of 16 mice each. Mice from groups 1 and 2 were subcutaneously challenged with 10^5 tachyzoites of the Nc-Spain7 isolate at midgestation (days 7 after mating), while mice from group 3 were left unchallenged and received a culture medium inoculation (26). Two days after infection, all mice from group 1 were given the BKI-1294 (50 mg/kg/day emulsified in 100 μl corn oil) for a period of 6 days by gavage once a day, while the group 2 mice received corn oil alone. Prior to gavage, the corn oil-drug mixture was heated to 37°C to enhance solubility of the drug. Group 3 remained untouched. Pregnancy was confirmed between days 15 and 18 of gestation, and pregnant mice were then allocated into single cages to give birth on days 20 to 22 and to rear their pups for an additional 30 days. During this time, nonpregnant mice were maintained in cages of 3 to 5. Nonpregnant mice, dams, and their offspring were evaluated for clinical signs of disease twice a day from the day of birth to day 30 postpartum (p.p.) according to the scoring scheme as above. Data on the number of female mice that became pregnant, litter size (number of delivered pups per dam), early pup mortality (number of full-term dead pups from birth until day 2 p.p.), postnatal mortality (number of

TABLE 2 IC_{50} of BKI-1294 in *N. caninum* and *T. gondii* strains[a]

Strain	IC_{50} (nM)
N. caninum Nc-Liv	360 ± 50
N. caninum Nc-Spain7	270 ± 20
T. gondii RH	20 ± 10
T. gondii ME49	220 ± 60
T. gondii RH CDPK1-wt	480 ± 100
T. gondii RH CDPK1-G128 M	2,260 ± 400

[a] HFF monolayers were treated with different concentrations of BKI-1294 or DMSO as a solvent control, infected with 5×10^4 tachyzoites, and incubated for 3 days. Cells were harvested, parasite load was determined, and IC_{50}s were calculated as described.

dead pups from day 3 to 30 p.p.), and clinical signs in dams and nonpregnant mice were recorded. Dams were weighed at days 15 and 30 p.p., and neonates were weighed every second day from day 15 p.p. onwards until the end of the experiment (day 30 p.p.). Nonpregnant mice were weighed once a week. Surviving dams, nonpregnant mice, and pups were euthanized in a CO_2 chamber latest at 30 days p.p. (approximately day 44 p.i.). Blood from dams and nonpregnant mice was recovered by cardiac puncture, and serum samples were obtained to assess humoral immune responses.

Analysis of biological samples from *in vivo* experiments. To quantify the parasite load in brains, DNA purification was performed employing the DNeasy blood and tissue kit (Qiagen, Basel, Switzerland) 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 to 5 ng/μl with sterile DNase-free water. Quantification of parasite loads in the brains of nonpregnant mice and dams was done as described previously (10, 27). Seropositivity for *N. caninum* was assessed as described previously (28, 29).

Statistics. Fifty percent inhibitory concentrations (IC_{50}s) were calculated after the logit log-transformation of the relative growth (RG; control = 1) according to the formula $\ln(RG/[1 - RG]) = a \times \ln(\text{drug concentration}) + b$ and subsequent regression analysis by the corresponding software tool contained in the Excel software package (Microsoft, Seattle, WA, USA). Statistical analysis of the parasite burdens in brains was done using the Kruskal-Wallis test. Comparison of positive and negative animals was performed using the chi-square test. All analyses were performed using the software package R (30).

RESULTS

***N. caninum* and *T. gondii* infections display similar sensitivities to treatment with BKI-1294 *in vitro*.** Different concentrations of BKI-1294 were added to *in vitro* cultures of *N. caninum* Nc-Liv and Nc-Spain7, two wild-type *T. gondii* strains (RH and ME49), and the two transgenic *T. gondii* strains (CDPK1_wt and CDPK1_G128M). The drug was added to HFF monolayers just prior to inoculation of tachyzoites. This resulted in IC_{50}s ranging from 20 nM (*T. gondii* RH) up to 360 nM (*N. caninum* Nc-Liv) (Table 2). The *T. gondii* strain CDPK1_G128M overexpressing a plasmid-encoded CDPK1 enzyme with a mutated (G to M) gatekeeper residue at position 128 was much less susceptible compared to the *T. gondii* RH and ME49 wild-type strains. The corresponding transgenic wild-type cell line, expressing an ectopic copy of nonmutated CDPK1, also exhibited a slightly higher IC_{50} than the wild-type strains in accordance to previous findings (12).

Treatments of *N. caninum* and *T. gondii* infections with BKI-1294 interferes with the separation of daughter zoites and leads to the formation of multinucleated complexes. The effects of continued BKI-1294 treatments in already infected HFF monolayers were visualized under a light microscope and by TEM. *T. gondii* ME49 and RH tachyzoites were allowed to invade HFF monolayers for 2 h prior to addition of 2.5 μM BKI-1294 or the corresponding amount of DMSO. Inspection by light microscopy showed that in treated and untreated cultures, large parasitophorous vacuoles were formed, indicating that parasite replication took place. However, the interior of the vacuoles in BKI-1294-treated cultures exhibited clear alterations compared to parasitophorous vacuoles from cultures maintained in the absence of the drug (see Fig. 1A through D). In untreated HFF, large vacuoles containing numerous tachyzoites were present after 2 to 3 days (Fig. 1A and C) followed by egress of tachyzoites from day 3 onwards. In BKI-1294-treated cultures, parasitophorous vacuoles had also increased in size, but egress of tachyzoites was blocked, and the interior of the vacuoles was not filled with individual parasites but larger structures of unknown nature (Fig. 1B and D). Similar findings were obtained in cultures infected with *N. caninum* Nc-Liv and Nc-Spain7 (data not shown). These observations were in line with earlier findings on transgenic Nc-betaGal treated with BKI-1294. Thus, TEM was used for further visualization of these interior complexes.

Figures 1E and F show nontreated *T. gondii* ME49 and RH tachyzoites cultured in HFF in the absence of drug at day 3 p.i. Tachyzoites proliferate intracellularly within a parasitophorous vacuole, surrounded by a parasitophorous vacuole membrane, and mostly individual tachyzoites are seen. Upon treatment with BKI-1294, the first signs of an inhibition of separation of newly formed tachyzoites in the two *T. gondii* strains were already observed on day 2 of treatment, as seen for *T. gondii* ME49 in Fig. 1G, and complexes increased in size and number of nuclei on day 4 (Fig. 1H). Figure 1I shows a vacuole containing two complexes of *T. gondii* ME49 treated with BKI-1294 for 6 days, with the two complexes embedded in a granular vacuolar matrix. One of these complexes already exhibited clear signs of structural impairment such as extensive intracellular vacuolization, while the other one still had more intact features, with a higher number of detectable nuclei and morphologically intact mitochondria and apical conoids being formed and protruding from its body. Figure 1J shows a multinucleated complex formed by *N. caninum* Nc-Liv tachyzoites treated with BKI-1294 for 6 days. Similar features were observed when *T. gondii* RH tachyzoites were treated with BKI-1294 for 6 days (Fig. 2A and B). In contrast, BKI-1294 treatment of the *T. gondii* cell line CDPK1_G128M overexpressing a mutated gatekeeper residue with a transition from G to M at position 128 did not lead to the multinucleated complex phenotype as observed for the other cell lines (Fig. 2D). These tachyzoites underwent proliferation within a parasitophorous vacuole and separated in a normal manner, in contrast to their respective wild type, *T. gondii* CDPK_wt overexpressing the wild-type CDPK1 (Fig. 2C). However, *T. gondii* CDPK1_G128M exhibited a slightly altered phenotype with increased intracytoplasmic vacuole formation (Fig. 2D).

BKI-1294 treatment results in deregulated antigen expression in *N. caninum* and *T. gondii*. In order to investigate whether bradyzoite-specific gene expression was induced by BKI-1294, HFF monolayers were infected with Nc-Liv or *T. gondii* ME49, cultured for 2 days and treated with BKI-1294 or with DMSO as a solvent control. During the next 4 days, the infected cell layers were harvested, and mRNA levels of the bradyzoite-specific antigen BAG1 and the tachyzoite-specific antigen SAG1 were determined in relation to Gra2 mRNA, which is expressed in bradyzoites and tachyzoites. After 2 and 4 days, BAG1 levels were

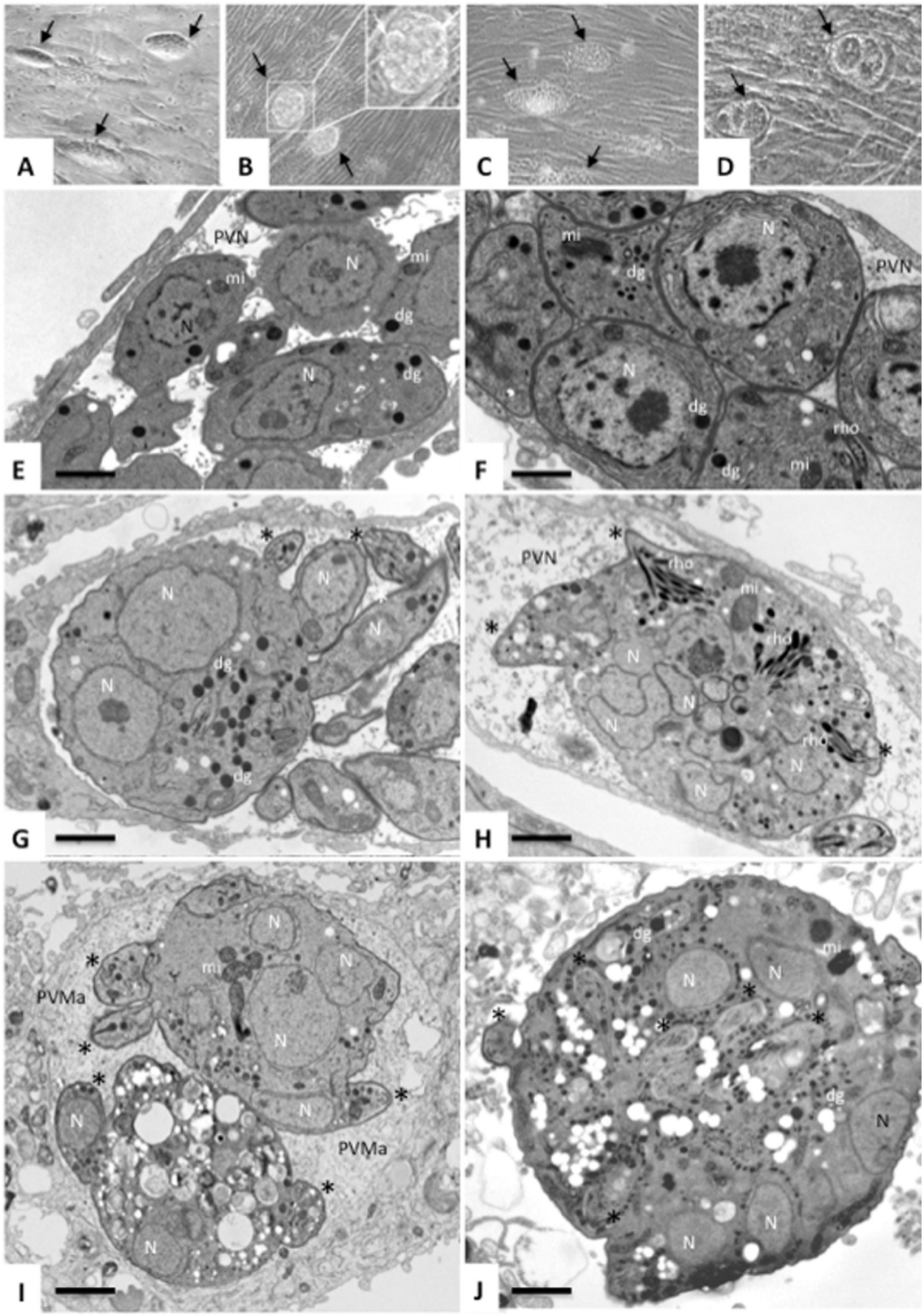

FIG 1 Effects of BKI-1294 treatment on intracellular *T. gondii* and *N. caninum* tachyzoites. (A to D) Phase contrast images of *T. gondii* ME49 (A, B) and RH (C, D) cultured in HFF in the absence (A, C) or presence (B, D) of BKI-1294. Arrows point toward parasitophorous vacuoles. (E, F) Electron micrographs of *T. gondii* ME49 and RH tachyzoites, respectively, cultured in HFF in the absence of the drug for 3 days. Tachyzoites exhibit mostly single nuclei (N), and rhoptries (rho), dense granules (dg), and mitochondria (mi) are visible. The interior of the parasitophorous vacuole is filled with a parasitophorous vacuole tubular network (PVN). (G, H, and I) Transition of *T. gondii* ME49 tachyzoites into multinucleated complexes upon exposure to BKI-1294 for 2 (G), 4 (H), and 6 (I) days. Emerging apical complexes are marked with an asterisk (*), and multiple nuclei are visible (N), as well as rhoptries (rho) and dense granules (dg). Note that at 6 days of BKI-1294 treatment, the matrix of the parasitophorous vacuole becomes more granular (PVMa). (J) Intracellular *N. caninum* Nc-Liv complex formed after 6 days of BKI-1294 treatment. (E) Bar = 1 μm; (F) bar = 0.8 μm; (G) bar = 1.2 μm; (H) bar = 1.8 μm; (I) bar = 2.5 μm; (J) bar = 2.1 μm.

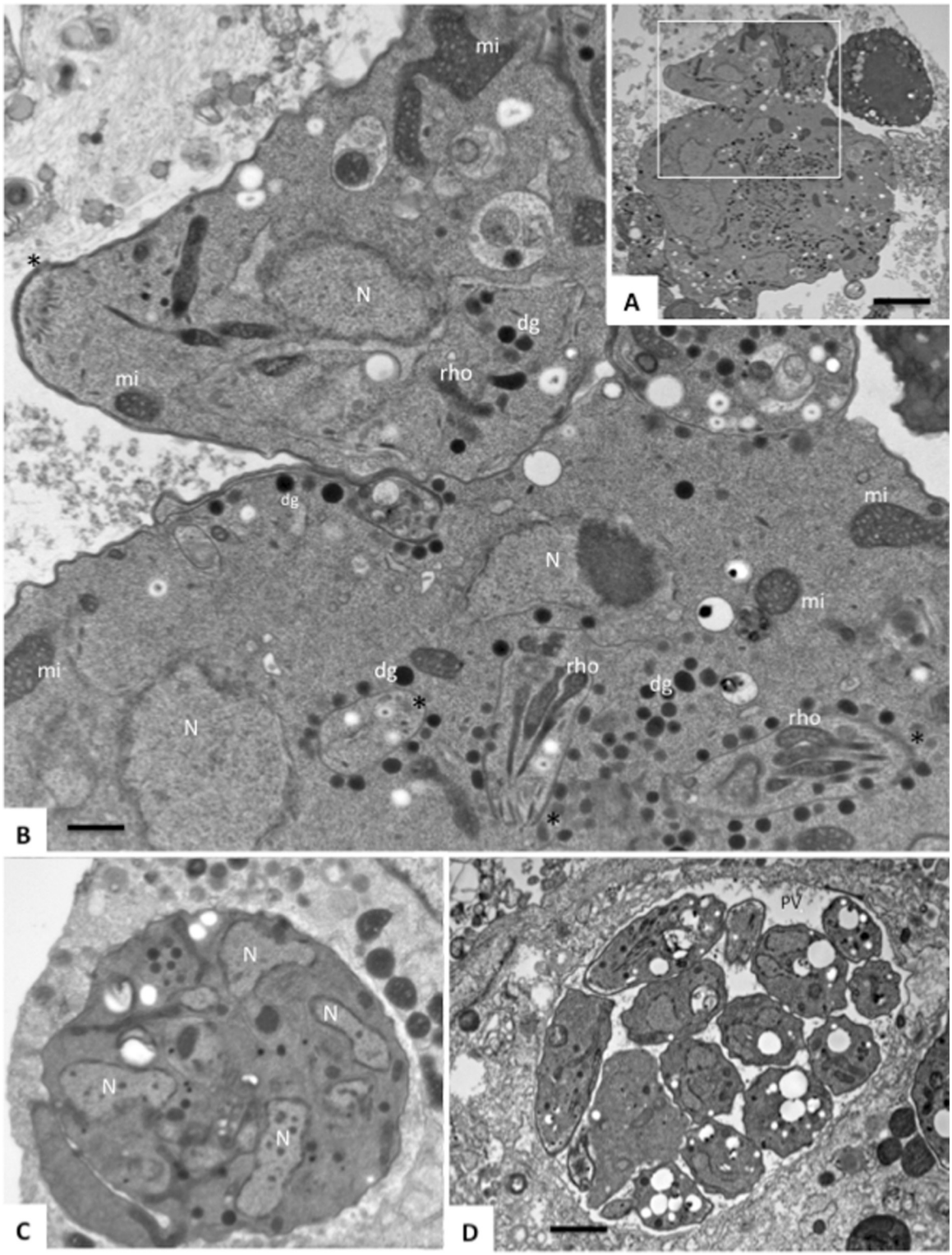

FIG 2 Complex formation of wild-type *T. gondii* RH and CDPK1_wt but not *T. gondii* CDPK1_G128M upon exposure to BKI-1294. (A) Low-magnification view of a large multinucleated complex formed by *T. gondii* RH; (B) the boxed area is shown enlarged. (C) *T. gondii* CDPK1_wt treated with BKI-1294. (D) No complexes are formed upon BKI-1294 treatment of *T. gondii* CDPK1_G128M. N, nucleus; mi, mitochondria; rho, rhoptry; dg, dense granules. (A) Bar = 2.5 μm; (B) bar = 0.6 μm; (C) bar = 2 μm; (D) bar = 1 μm.

TABLE 3 BAG1 and SAG1 gene expression during treatment with BKI-1294[a]

Transcript	Relative mRNA level	
	N. caninum Liverpool	*T. gondii* ME49
BAG1		
Day 2	13 ± 4	729 ± 220
Day 4	7 ± 1	413 ± 77
SAG1		
Day 2	1.4 ± 0.4	17 ± 1
Day 4	1.3 ± 0.2	31 ± 2

[a] Confluent HFF monolayers grown in 6-well plates were infected with 2.5×10^4 Nc-Liv or TgME49 tachyzoites. Treatment with 5 μM BKI-1294 or DMSO (control) was initiated 2 days postinfection. After different time points, the adherent cells were harvested for RNA extraction. mRNA levels of BAG1, SAG1, and Gra2 were quantified by real-time RT-PCR. Mean values (±SE) from four replicates were assessed, and expression levels of BAG1 (bradyzoite specific) or SAG1 (tachyzoite specific) relative to the amount of GRA2 (overall) mRNA were given as multiples of the corresponding DMSO control values (MV ± SE for quadruplicates).

strongly increased in cells infected with *N. caninum* Nc-Liv or *T. gondii* ME49 treated with BKI-1294. SAG1 levels remained constant in Nc-Liv and were even increased in *T. gondii* ME49 upon BKI-1294 treatment (Table 3). Conversely, BAG1 expression was reduced in *T. gondii* CDPK1_G128M compared to its isogenic strain overexpressing wild-type CDPK1 (Fig. 3).

Moreover, the expression of tachyzoite-specific antigen SAG1 and the bradyzoite marker BAG1 in BKI-1294-treated and untreated *T. gondii* ME49 and *N. caninum* Nc-Liv was investigated by immunofluorescence (Fig. 4 and 5). In addition, a second bradyzoite marker recognized by the mAbCC2 (18, 31) was also studied. *Toxoplasma* (Fig. 4A through D and M through P) and *Neospora* (Fig. 5A through D and M through P) control cultures maintained in the absence of the drug are shown at 3 days postinfection, as they were normally lysed completely after 4 to 5 days. In these controls, SAG1 expression was always evident on tachyzoites localized in large parasitophorous vacuoles, while staining with antibodies against BAG1 and the mAbCC2 were either sparse or nonexistent, indicating that in nontreated cultures the protein expression levels of these antigens were low. In contrast, BKI-1294 treatment led to increased BAG1 protein expression and mAbCC2 labeling in *T. gondii* (Fig. 4E through L and Q through T) and *N. caninum* (Fig. 5E through L and Q through T). Most notably, BAG1 (Fig. 4A through L and Fig. 5A through L) was detected already after 4 days of BKI-1294 treatment, and staining intensity increased consistently with time. Within these multinucleated complexes, SAG1 and BAG1 were distributed unevenly, and in many instances, staining of BAG1 antigen occurred in regions with lower SAG1 expression. MAbCC2 labeling was comparatively assessed to staining of complexes with a polyclonal antiserum raised against crude *T. gondii* (Fig. 4M through T) and *N. caninum* antigens (Fig. 5M through T), and staining intensity also increased with time.

Treatment of pregnant mice with BKI-1294 has no side effects and protects pups from vertical transmission of *N. caninum*. In order to investigate whether BKI-1294 affected pregnancy and the number of offspring, pregnant mice were treated with BKI-1294 (50 mg/kg/day) during 8 days or with the vehicle (CMC, 0.5%) alone. The numbers of offspring in the different treatment groups were virtually identical. Fifty-four offspring were born in the BKI-1294 treatment group, and 46 pups were born in the control group (with 2 stillborn pups in each group). No negative impact on the further development of offspring mice was detected during at least 1 month after birth (data not shown). Thus, BKI-1294 treatment did not affect pregnancy outcome.

These findings prompted us to proceed to experimental treatments of infected mice with BKI-1294. In a first experiment, nonpregnant mice and pregnant mice were infected at the same time (7 days postmating) by intraperitoneal injection of 2×10^6 *N. caninum* Nc-Liv tachyzoites. Two days later, BKI-1294 treatment was initiated by oral gavage (50 mg/kg/day for 8 days) formulated in 0.5% CMC, while the control groups received CMC only. None of the mice died during the 2-week period following infection, but 4 out of 6 dams in the control group exhibited mild symptoms such as ruffled coat and moderate weight loss, while in all other groups, no clinical signs were detected. After the dams gave birth, all mice including offspring were euthanized, and the effects of the treatment were evaluated. Thirty-nine pups were born in the control group, and 27 pups were born in the BKI-1294-treated group (the difference was not statistically sig-

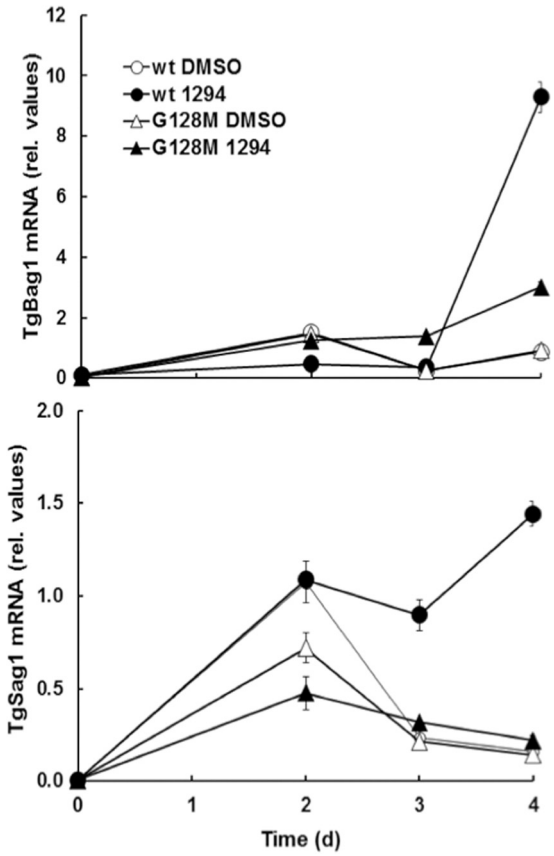

FIG 3 Quantification of BAG1 and SAG1 gene expression during treatment with BKI-1294. Confluent HFF monolayers grown in 6-well plates were infected with 2.5×10^4 tachyzoites of *T. gondii* RH overexpressing CDPK1_G128M or wild-type CDPK1. Treatments with 5 μM BKI-1294 or DMSO (control) were initiated 2 days postinfection. After different time points, the adherent cells were harvested for RNA extraction. mRNA levels of BAG1, SAG1, and GRA2 were quantified by real-time RT-PCR. Mean values (±SE) from four replicates were assessed, and expression levels of BAG1 (bradyzoite specific) or SAG1 (tachyzoite specific) relative to the amount of GRA2 (overall) mRNA were given as multiples of the corresponding DMSO control values (MV ± SE for quadruplicates).

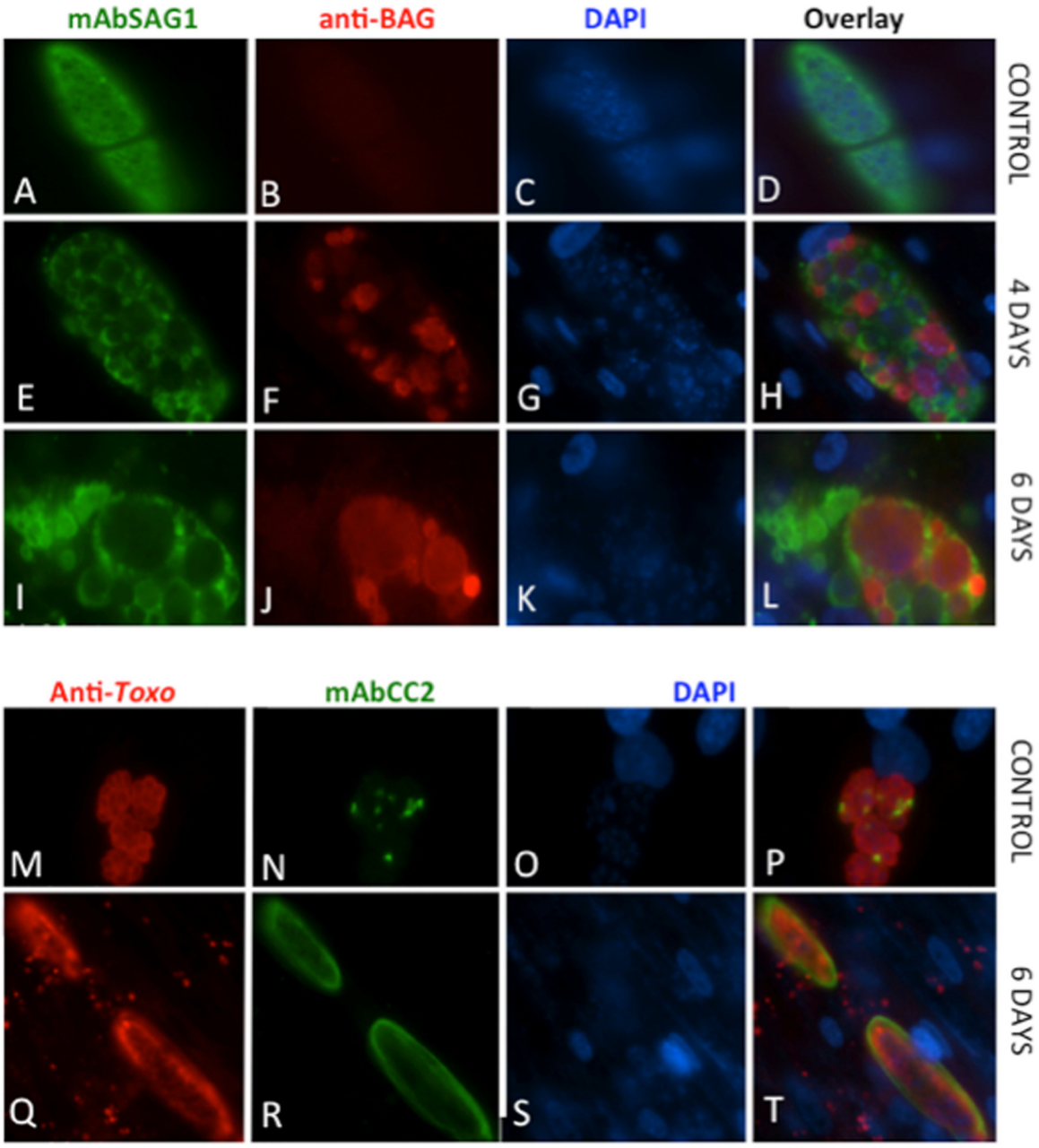

FIG 4 BKI-1294 treatment of *T. gondii* ME49 cultured in HFF induces BAG1 protein expression and increased staining with mAbCC2. Controls (A to D and M to P) were fixed and processed after 3 days, and BAG1 labeling is shown after 4 and 6 days of BKI-1294 treatment. Staining with mAbCC2 is shown after 6 days of treatment (E to L and Q to T). Note the increased staining of the two bradyzoite markers in BKI-treated cells.

nificant). All infected mice were seropositive for *N. caninum*. All control mice (nonpregnant and dams) were tested PCR-positive in the brain, whereas only one nonpregnant female and one dam were positive in the BKI-1294-treated groups. Of the 27 pups born in the BKI-1294-treated group, all brains were real-time PCR-negative, while in the 39 pups born in the control group, 21 tested positive. These differences were highly significant (Table 4).

In order to assess the reproducibility of these findings using another virulent *N. caninum* isolate, another mode of infection, and another formulation of the compound, a second experiment was performed according to the recently standardized infection model established by Arranz-Solís et al. (26). Mice were mated as described above and infected 7 days postmating by subcutaneous injection of 10^5 *N. caninum* Nc-Spain7 tachyzoites. Two days later, BKI-1294 treatment was initiated by oral gavage (50 mg/kg/day formulated in corn oil for a period of 6 days). Pregnant and nonpregnant mice were separated and analyzed separately as described. All infected mice were seropositive for *N. caninum*. None of the nonpregnant mice exhibited clinical signs of neosporosis during the 3 weeks following infection. After the dams gave birth, offspring and dams were maintained for 30 days in order to investigate how the treatments had an impact in the long term during the postnatal phase in dams and offspring. Whereas 5 of

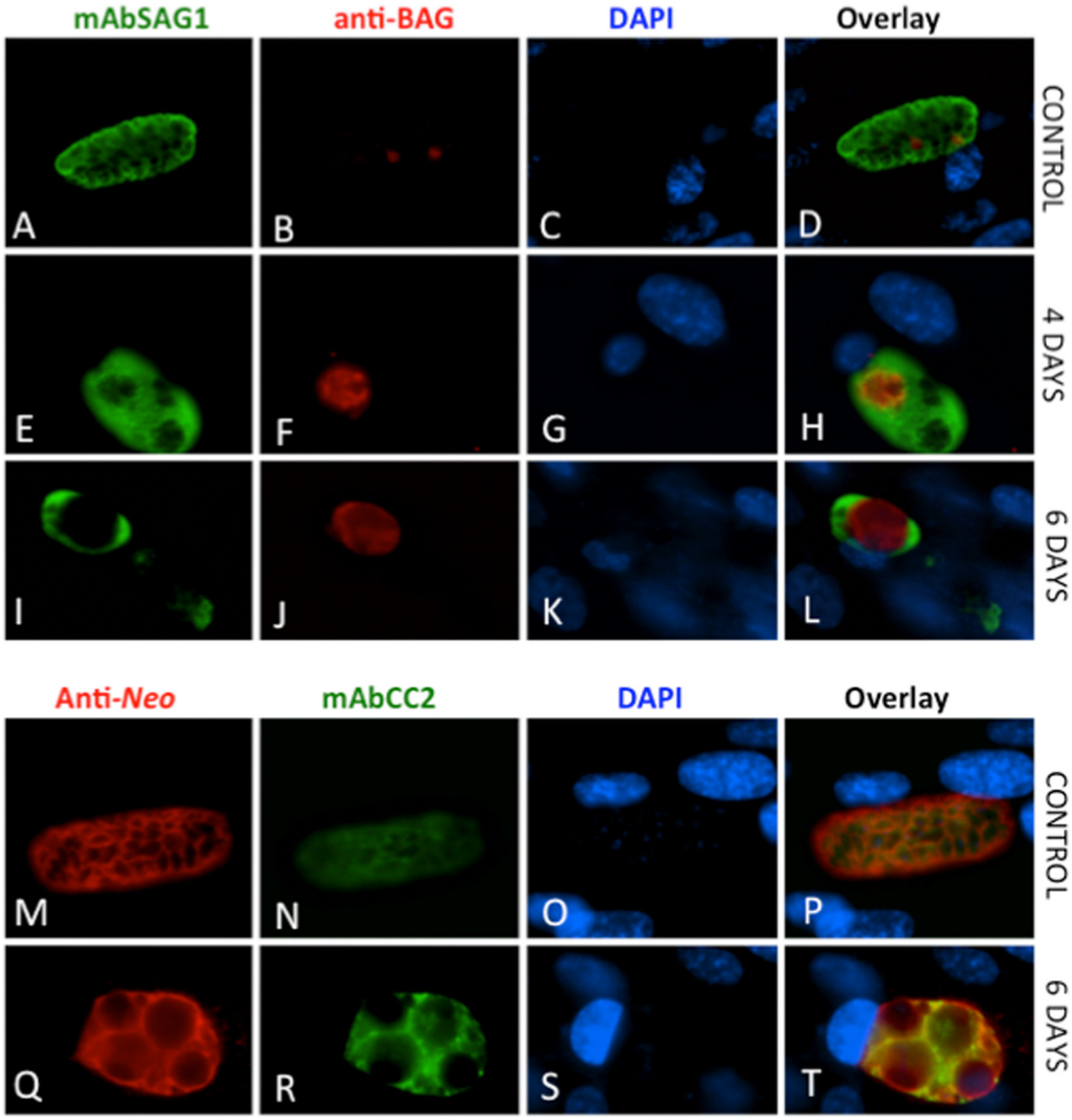

FIG 5 BKI-1294 treatment of *N. caninum* Nc-Liv cultured in HFF induces BAG1 protein expression and increased staining with mAbCC2. Controls (A to D and M to P) were fixed and processed after 3 days, and BAG1 labeling is shown after 4 and 6 days of BKI-1294 treatment. Staining with mAbCC2 is shown after 6 days of treatment. Note the increased staining of the two bradyzoite markers.

8 dams of the placebo control group showed clinical signs and one died before the end of the experiment, only 2 of 10 mice from the treated group showed mild clinical signs and none of them died before the end of the experiment (Table 5). Cerebral parasite loads in nonpregnant mice and dams as quantified by PCR were significantly lower in the treated group, demonstrating that BKI-1294 treatment has essentially prevented cerebral infection with *N. caninum* Nc-Spain7 (Fig. 6A).

After 30 days p.p., all surviving mice including offspring were euthanized, and the effects of BKI-1294 exposure were evaluated with regard to cerebral infection in surviving dams and nonpregnant mice and pups. The number of pups per dam was not significantly affected by the treatment. In the two groups, two pups died immediately after birth. Later, 40 of the remaining 41 pups died in the control group but only 4 of 56 in the treated group (see Fig. 6B). Based on previous results (26), the dead pups were considered PCR-positive for *N. caninum*. After 30 days, all survivors were tested by PCR for the presence of *N. caninum* in their brains. The last survivor from the control group was brain positive, as well as 5 of 52 surviving pups from the treated dams. Thus, mortality and transmission of *N. caninum* to pups was strongly reduced by BKI-1294 treatment of the dams (Fig. 7). The differences were highly significant (Table 5).

DISCUSSION

In apicomplexan parasites, calcium-mediated signaling plays a major role in a variety of crucial activities, including events that ensure progression in the life cycle such as secretion, gliding mo-

TABLE 4 Effects of BKI-1294 treatments on cerebral *N. caninum* Nc-Liv infection in nonpregnant mice, dams, and pups[a]

Parameter	BKI-1294	Placebo control
Nonpregnant females (no./total no.)		
Total no.	6	6
Seropositive	6/6	6/6
N. caninum positive	1/6[b]	6/6
Dams and pups (no./total no.)		
Total no. of dams	6	6
Seropositive dams	6/6	6/6
N. caninum-positive dams	1/6[b]	6/6
Total no. of pups	27	39
N. caninum-positive pups	0/27[c]	21/39
Stillborn pups	6/27[d]	8/39

[a] Mice were treated with BKI-1294 in CMC or with CMC alone as a placebo control, infected with Nc-Liv, and sacrificed as described in Materials and Methods. Adults and pups were tested for the presence of *N. caninum* in their brains with a specific real-time PCR. Respective numbers of animals in control and 1294-treated groups were compared by chi-square tests.
[b] $P < 0.005$.
[c] $P < 0.001$.
[d] $P > 0.1$.

TABLE 5 Effects of BKI-1294 treatments on clinical signs, mortality, fertility, and cerebral *N. caninum* Nc-Spain7 infection in nonpregnant mice, dams, and pups[a]

Parameter	BKI-1294	Placebo control
Nonpregnant females (no./total no.)		
Total no.	6	8
Clinical signs	0/6	0/8
Mortality	0/6	0/8
Seropositive	6/6	8/8
N. caninum positive	1/6[b]	8/8
Dams and pups (no./total no.)		
Total no. of dams	10	8
Clinical signs	2/10[c]	5/8
Mortality	0/10[d]	1/8
Seropositive dams	10/10	8/8
N. caninum-positive dams	3/10[b]	8/8
Pregnancy rate	10/16	8/16
Total no. of pups	58	43
Litter size avg (no. of pups/no. of dams)	58/10[d]	43/8
Neonatal mortality[e]	2/58[d]	2/43
Postnatal mortality[f]	4/56[g]	40/41
N. caninum-positive pups	9/58[g]	43/43

[a] Mice were treated with BKI-1294 in corn oil or with corn oil alone as a placebo control, infected with Nc-Spain7, and sacrified as described in Materials and Methods. Adults and surviving pups were tested for the presence of *N. caninum* in their brains with a specific real-time PCR. Pups that had died before the end of the experiment were considered *N. caninum* positive. Respective numbers of animals in control and 1294-treated groups were compared by chi-square tests.
[b] $P < 0.005$.
[c] $P < 0.1$.
[d] $P > 0.1$.
[e] Proportion of pups born dead or that died within the 2 first days postpartum.
[f] Proportion of pups dead from days 3 to 30 postpartum.
[g] $P < 0.001$.

tility, and entry into host cells (32), as well as intracellular proliferation, cytokinesis, and stage differentiation (33). Calcium also activates CDPKs, which are found in plants, ciliates, and in apicomplexan parasites but not in mammalian cells (34). They are thus promising targets for chemotherapeutical intervention. In *T. gondii*, it was shown that CDPK1, a member of the serine/threonine kinase family, regulates the calcium-dependent pathway of microneme secretion and governs cell attachment (35), migration, and microneme secretion (36). The ATP-binding site of *T. gondii* CDPK1, but also of other related apicomplexans, such as *N. caninum* and *Cryptosporidium parvum*, is characterized by a small glycine gatekeeper residue (10, 37), while most mammalian kinases have larger residues such as methionine or phenylalanine at that position. Selective and potent inhibitors have been generated that take advantage of the enlarged space in the CDPK1 ATP-binding site (37–39). Benzoyl benzimidazole-based selective inhibitors and BKIs based on a pyrazolopyrimidine scaffold can block *T. gondii* CDPK1 and affect host cell invasion (40–42).

Here, we focus on the pyrazolopyrimidine compound BKI-1294. BKI-1294 was reported earlier to inhibit host cell invasion of transgenic *T. gondii* RH and *N. caninum* tachyzoites expressing beta-galactosidase, with IC_{50}s of 137 and 32 nM, respectively, if the compound was added to the cultures at the time point the parasites were allowed to invade their host cell (10, 39). We show here that BKI-1294 also exhibited *in vitro* activity against the virulent *N. caninum* isolates Nc-Liv and Nc-Spain7 (IC_{50}s = 360 and 270 nM, respectively) and similarly against the cyst-forming *T. gondii* ME49 strain, while *T. gondii* RH tachyzoites were much more susceptible (IC_{50} = 20 nM). The value we obtained for *T. gondii* RH inhibition (20 nM) differs substantially from those reported previously (39). However, it is important to note that in this study parasite loads were quantified by real-time PCR while in earlier reports transgenic parasites expressing beta-galactosidase were used, and some degree of variation may be attributed to different culture conditions, host cells, and slight variations in protocols for parasite isolation prior to infection of HFF. In addition, strains and isolates that have been maintained in culture for extended periods of time may exhibit various drug susceptibilities. However, in accordance with an earlier report (12), the sensitivity to BKI-1294 was entirely lost in *T. gondii* CDPK1_G128M, which overexpresses a mutated *Tg*CDPK1. Crystallographic confirmation of the interaction between BKI-1294 and *Nc*CDPK1 showed

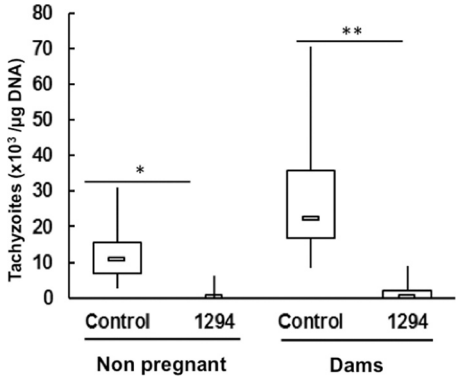

FIG 6 Mice treated with BKI-1294 have a lower cerebral parasite load. BALB/c mice were infected with Nc-Spain7 tachyzoites and subsequently treated with BKI-1294 in corn oil (1294) or corn oil only (control) as detailed in Materials and Methods. Nonpregnant mice were euthanized 3 weeks p.i., and dams were euthanized 30 days postpartum. After euthanasia, brains were collected and the amount of tachyzoites was determined by quantitative PCR (*, $P < 0.005$; **, $P < 0.001$; Kruskal-Wallis test).

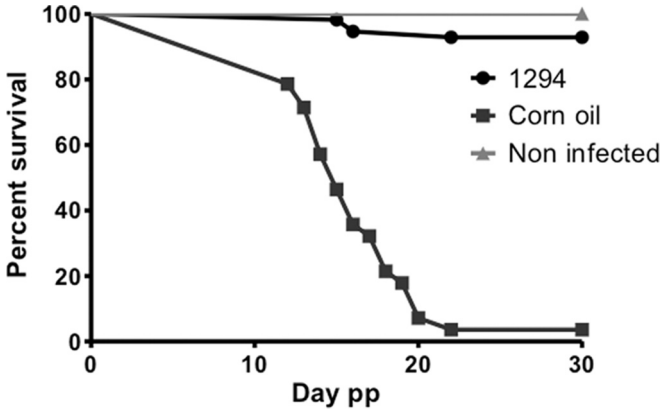

FIG 7 Offspring from BKI-1294-treated mice exhibit a similar survival rate as mice from uninfected and nontreated mice. Survival curves of pups during 30 days postpartum (pp) of BKI-1294-treated (1294) and placebo-treated (corn oil only) groups, each of which was infected with Nc-Spain7 isolates of *N. caninum*. A group that was not infected and remained untreated with any compound (noninfected) exhibited 100% survival.

that binding was entirely consistent with that previously determined for the *Tg*CDPK1 homologue, which is 96% identical and differs in the active site by only one residue (10). This clearly indicates that CDPK1 represents the main target for BKI-1294 in *T. gondii* and *N. caninum*.

However, BKIs might also affect other targets. We have recently shown that exposure of intracellular Nc-betaGal tachyzoites to BKI-1294 did not only inhibit egress but led to the formation of large multinucleated complexes within 5 to 9 days of culture, which can be visualized by TEM (10). Here, we demonstrate that similar multinucleated complexes occurred upon exposure of *T. gondii* ME49 and *T. gondii* RH tachyzoites if treatment with BKI-1294 was initiated after host cell invasion has already taken place. These complexes contained several nuclei and protrusions carrying features of the apical complex such as conoid, dense granules, rhoptries, and micronemes. Thus, DNA replication and daughter nucleus formation took place; however, cytokinesis of the daughter cells was not completed. Many complexes were still clearly viable after 6 days of *in vitro* treatment, while others showed signs of deterioration. However, BKI-1294 treatment of the resistant *T. gondii* cell line CDPK1_G128M did not lead to the multinucleated complex phenotype seen in the wild-type cell lines, suggesting that the observed effect may have some association with CDPK1 inhibition. The various metabolic pathways for which *Tg*CDPK1 and *Nc*CDPK1 are associated have not been fully defined. We have however shown that *Tg*CDPK1 was highly localized in the cytoplasm but also found in the nucleus (12). Nuclear localization suggests that the *Tg*CDPK1 may be responsible for phosphorylating nuclear proteins, potentially relaying a shift in transcription associated with invasion and/or other yet to be defined functions associated with cytokinesis of the daughter cells. We also show that intracellular *Neospora* and *Toxoplasma* exposed to BKI-1294 treatment exhibited markedly elevated BAG1 transcript levels and increased labeling by antibodies directed against BAG1 and by the mAbCC2, another marker for bradyzoite stage differentiation (18).

Abnormally enlarged parasites and upregulation of bradyzoite-specific gene expression upon protein kinase inhibitor treatments have been reported by others as well (43, 44). Moreover, BKI analogs 1NM-PP1, 3MB-PP1, and 3BrB-PP1 increased BAG1 gene promoter activity (45). Protocols for *in vitro* induction of tachyzoite-to-bradyzoite stage conversion such as high pH and CO_2 depletion also cause defects in cytokinesis and asynchronous cell division (46). However, rather than postulating that BKI-1294 treatment causes bradyzoite stage conversion, we hypothesize that exposure to BKI-1294 leads to deregulated gene expression, which does not necessarily interfere in DNA replication but severely hampers cytokinesis and thus the separation of newly formed daughter zoites.

A secondary BKI target that is actually involved in cytokinesis has been defined (47), showing that mutations in the *T. gondii* mitogen-activated protein kinase-like 1 (*Tg*MAPKL-1; TGME49_312570) resulted in a 3.5-fold increased resistance of these parasites against the BKI 1NM-PP1. Treatment of *T. gondii* tachyzoites with the BKI 1NM-PP1 also led to enlarged, multinucleated parasites that were severely impaired in coordinated cell cycle progression. Furthermore, a single mutation in the gatekeeper residue of this kinase restored the inhibitory effect of the BKI 1NM-PP1 (13). Comparison of the genomes of *N. caninum* and *T. gondii* reveals the presence of a *Tg*MAPKL-1 orthologue in *N. caninum* (*Nc*MAPK-3; NCLIV_056080), with a highly conserved kinase domain of 80% identity. However, whether the BKI-1294 also inhibits *Tg*MAPKL-1 and *Nc*MAPK-3 activities is not known to date. In the *N. caninum* genome, other enzymes with small gatekeeper residues were identified that BKI-1294 may potentially inhibit, including *Nc*CamK (NCLIV_046430) with an alanine gatekeeper, an *Nc*AGC-like kinase (NCLIV_016060) with a serine gatekeeper, and an *Nc*ROP-like kinase (NCLIV_030990) with a threonine gatekeeper (10). Engagement of multiple parasite targets may be a potential advantage, especially since BKI-1294 was shown to be parasite specific and exhibits very low toxicity in mammalian cells and no off-target inhibition of small gatekeeper (threonine) human protein kinases (39, 48).

To provide a proof of concept that the BKI-1294 can prevent *Neospora*-related abortion in cattle, we investigated the effects of BKI-1294 treatments in a pregnant mouse model. The first *in vivo* study showed that oral application of BKI-1294 at a dosage of 50 mg/kg/day for a period of 8 days in pregnant, but noninfected, mice had no negative impact, neither on litter size, neonatal mortality, nor subsequent development of the offspring during 1 month postpartum. This is in line with earlier studies demonstrating that mice receiving 100 mg/kg BKI-1294 twice daily for 5 days did not show any signs of toxicity or weight loss, and no alterations in tissue histology, metabolic enzymes, and complete blood counts (48). Subsequently, the two *in vivo* studies in *N. caninum* Nc-Liv- and Nc-Spain7-infected mice showed that BKI-1294 prevented vertical transmission of *N. caninum* from dams to pups without impairing vitality and fertility of the dams. Due to the close phylogenetic relationship between *N. caninum* and *T. gondii* and the similarities of the effects in the two parasites exerted by BKI-1294 treatments, the current results in the pregnant model for *Neospora* infection should motivate testing the drug in a pregnant *T. gondii* mouse model to extend these results to that parasite.

Although BKI-1294 was surprisingly efficacious, the outcome of these experiments is not a surprise as such. The *in vivo* efficacy of this compound against other apicomplexan parasites in mice has been previously reported, such as activity against cryptosporidiosis (8) and experimental toxoplasmosis inflicted by the viru-

lent *T. gondii* RH strain (49). The BKI 1NM-PP1 also inhibited *T. gondii* RH strain infection in mice, although only when given in high doses by i.p. injection prior to infection via the intraperitoneal route (50). A similar BKI series was assessed *in vivo* against the cyst-forming type II strain *T. gondii* Pru, and these compounds prolonged survival and decreased the numbers of brain cysts at 30 days p.i. (38). However, in this study, compound administration was started 1 day prior to infection and lasted for a period of 10 days. In contrast, in our experiments the BKI-1294 was administered 48 h after infection, hence, when a robust infection and dissemination of the parasite in the mice has already taken place. This clearly indicates that BKI-1294 probably exerts effects *in vivo* that go far beyond its expected impact on host cell invasion as the primary target, and this may be well in line with our *in vitro* observations. Thus, those effects seen *in vitro* may also affect parasite viability *in vivo* since BKI-1294 achieved therapeutic concentrations in mice that mimic those of our *in vitro* treatment experiments; the mean serum concentration after administration of 40 mg/kg of body weight for 6 consecutive days in mice resulted in a serum concentration of 6.3 ± 1.8 μM, and the therapeutic concentrations in the brain are about 30% of the serum concentration (49). Pharmacokinetic studies showed that hepatic metabolism of BKI-1294 becomes saturated with repeated administration and increased dosage (48). The fact that these therapeutic concentrations *in vivo* are sufficiently high to potentially induce the formation of rather long-lived multinucleated complexes in an infected animal may have important implications for the formation of a sustainable immunological response. For instance, the Nc-Spain7-infected dams were maintained for a period of 38 days following BKI-1294 treatment, and still only minimal cerebral parasite loads were noted. Such an immune response, directed against antigens expressed by parasites blocked in the process of cytokinesis but still viable, may be effective for extended periods of time after the end of dosing, and may well contribute to the excellent efficacy of BKI-1294. This hypothesis of an enhanced antigen expression causing a strong immune response during BKI-1294 therapy and leading to better outcomes in immunocompetent animals will be the subject of future studies.

In conclusion, the broad-spectrum anti-apicomplexan compound BKI-1294 exhibits excellent potency and specificity and does not cause toxicity in pregnant mice. Its impact on CDPK1 and host cell invasion is undisputed, but potentially other targets related to the completion of cytokinesis may also be affected. Intracellular parasites exposed to BKI-1294 exhibit a deregulated gene expression and build up multinucleated and rather long-lived complexes. Further investigations will focus on the expression profile of these complexes and their role in the immune response in BKI-1294-treated animals.

ACKNOWLEDGMENTS

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