**Bumped Kinase Inhibitor 1294 Treats Established Toxoplasma gondii Infection**

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**Toxoplasma gondii** is a unicellular parasite that causes severe brain and eye disease. Current drugs for **T. gondii** are limited by toxicity. Bumped kinase inhibitors (BKIs) selectively inhibit calcium-dependent protein kinases of the apicomplexan pathogens **T. gondii**, cryptosporidia, and plasmodia. A lead anti-**Toxoplasma BKI**, 1294, has been developed to be metabolically stable and orally bioavailable. Herein, we demonstrate the oral efficacy of 1294 against toxoplasmosis in vivo.

**T. gondii** is a highly infectious unicellular parasite that causes debilitating brain and eye disease. Approximately one-third of humans have been infected with **T. gondii**. In the great majority of infections, **T. gondii** establishes latent brain infection. If the immune system is compromised, **T. gondii** emerges, causing encephalitis. In immunocompetent individuals, virulence varies geographically. **T. gondii** is a prominent cause of blindness in South America, and cases of severe disseminated disease with respiratory failure have occurred in French Guiana (1, 2). The current first-line drug regimens target the **T. gondii** folate pathway. These regimens require long durations of drug treatment and are frequently not tolerated due to allergic reactions or hematologic toxicity. Herein we describe the in vivo anti-**T. gondii** efficacy of the bumped kinase inhibitor (BKI) 1294 that was selected from a library of BKIs for its outstanding potency, selectivity, and pharmacokinetics. Moreover, these experiments show that BKIs are orally effective against established **T. gondii** infection.

BKIs are a class of anti-**T. gondii** compounds that selectively target the **T. gondii** calcium-dependent protein kinase 1 (TgCDPK1), a member of the serine/threonine protein kinase family. TgCDPK1 regulates the calcium-dependent pathway of **T. gondii** microneme secretion and is required for gliding motility, host-cell invasion, and egress (3). As anticipated, pharmacological inhibition of TgCDPK1 blocks host-cell invasion, thereby inhibiting **T. gondii** growth (4, 5). Recently, Sugi et al. found that mutations in the **T. gondii** mitogen-activated protein kinase 1 (TgMAPK1) conferred up to 3.5-fold resistance to the BKI 1NM-PP1, suggesting that TgMAPK1 is a secondary target (6). The target of 1294 is TgCDPK1, as demonstrated by an 11-fold resistance to 1294 caused by an amino acid substitution (G128M) at the “gatekeeper residue” of TgCDPK1 (7).

A key structural difference between TgCDPK1 and human kinases occurs at the gatekeeper residue in the ATP-binding pocket. TgCDPK1 contains a small glycine residue at this position, whereas human kinases have larger residues, threonine being one of the smallest. The additional space afforded by the glycine residue in TgCDPK1 has been exploited for the design of potent and selective ATP-competitive TgCDPK1 inhibitors (7, 8, 9). A pyrazolopyrimidine scaffold that binds in the ATP binding pocket was optimized for TgCDPK1 selectivity by placing a 6-alkoxy-2-naphthyl group at the C-3 position (Fig. 1). This bulky C-3 substituent sterically hindered by the larger gatekeeper residues found in human kinases. Further selectivity was accomplished by placing a 4-piperidinylmethylene group at the N-1 position. This substituent fully occupies the ribose-binding pocket in TgCDPK1 and forces the bulky C-3 group into a position that cannot be accommodated by human kinases (8). Pyrazolopyrimidine inhibitors with 6-alkoxy-2-naphthyl groups at the C-3 position and a 4-piperidinylmethylene group at the N-1 position are >15,000-fold more active against TgCDPK1 than the human kinases Src and Abl, with no inhibition of the human kinases at 20 μM. Src and Abl are two of the most likely off-target human kinases of BKIs because they have a relatively small threonine gatekeeper residue.

1294 possesses the above-mentioned N-1 and C-3 substituents that confer anti-**T. gondii** specificity as well as the N-methylation of the 4-piperidinyl-methylene substituent that provides metabolic stability (Fig. 1). 1294 achieves therapeutic concentrations in mice far above the in vitro **T. gondii** 50% inhibitory concentration (IC₅₀) of 140 nM. The mean (± standard deviation) serum concentration of 1294 at 40 mg/kg of body weight after 24 h in mice was 2 ± 1.6 μM, and the 24-h trough after 4 daily doses was 6.3 ± 1.8 μM. 1294 serum concentrations are further affected by nonlinear kinetics as evidenced by a 24.6-fold increase in the area under the curve (AUC) and a 31% increase in oral bioavailability to 81% when the oral dose was increased from 10 to 100 mg/kg. These findings suggest that the hepatic metabolism of 1294 becomes saturated with repeated administration and increased dose (10). 1294 attains therapeutic brain concentrations that are necessary for the treatment of toxoplasmosis. After 5 doses of 50 mg/kg/day in 2 uninfected 14-week-old female BALB/c mice, the brain concentrations of 1294 were 36% and 26% of the serum 1294 concentrations. 1294 was extracted from the homogenized brain and blood plasma sample with acetonitrile containing an internal standard and measured with liquid chromatography/
mass spectrometry (LC/MS). The percentage of 1294 in the brain was determined after adjustment for a 3% blood volume in the brain. Mice receiving 1294 at 100 mg/kg twice daily for 5 days did not show signs of toxicity or weight loss, and their tissue histology, metabolic enzymes, and complete blood counts were normal (10).

Based on the above pharmacokinetic parameters, 1294 was selected from a library of BKIs for further in vivo testing.

Here we describe the in vivo activity of 1294 against acute T. gondii in mice in 2 replicate experiments. Type I RH strain T. gondii tachyzoites (10^5) expressing yellow fluorescent protein were harvested from human foreskin fibroblasts, passed through a 3-μm-pore-size filter, and inoculated in a volume of 100 μl of phosphate-buffered saline (PBS) intraperitoneally (i.p.) into 4- to 5-week-old, 25-g female CF-1 mice. 1294 was dissolved in polyethylene glycol (PEG) 400 and administered by oral gavage 48 h after inoculation at concentrations of 100 and 30 mg/kg/day for 5 days. These doses were chosen based on the pharmacokinetics and in vitro IC_{50} of 1294 to evaluate the effect of full and partial anti-T. gondii activity. The control group received PEG 400 only. Groups consisted of 4 mice. Mice were euthanized on the eighth day and underwent peritoneal lavage with 3 ml of PBS (pH 7.4). A sample of 10 μl of peritoneal lavage fluid was examined in a hemocytometer using a Nikon Eclipse C1 fluorescence microscope with a fluorescent filter (excitation/emission 480/535 nm). In the control slides, 4 predetermined fields (total volume of 0.3 mm^3) were counted, and the number of T. gondii per ml was calculated. In the 30-mg/kg group, 4 fields (total volume of 0.4 mm^3) were counted. The entire field containing 10 μl of fluid was counted in the 100-mg/kg group. Tachyzoites were quantified per ml of fluid by 93% (1.25 log) as averaged from the 2 experiments. The 100-mg/kg dose almost completely eliminated T. gondii from the peritoneal fluid. In half of the mice tested in the 100-mg/kg group, there were no T. gondii detected. In the remaining half, there were 1 to 6 T. gondii per 10-μl sample. These results show that at 100 mg/kg, 1294 reduces the burden of infection far below the initial inoculum of 10^5 tachyzoites.

BKIs have broad activity against apicomplexan pathogens. 1294 is active against cryptosporidiosis in mice, blocks Plasmodium falciparum exflagellation and transmission to mosquitoes, and is effective against experimental toxoplasmosis (10, 11). Preceding, a different BKI (1NM-PP1) was shown to inhibit the virulent type I RH strain of T. gondii in mice when given in high doses i.p. prior to i.p. inoculation of T. gondii. However, i.p. administration of this compound after inoculation did not have an effect, and oral administration did not change the survival rates of mice (12). Subsequently, a similar series of BKIs were tested in vivo against the less virulent type II Pru strain of T. gondii. In this experiment, drugs were administered i.p. for 10 days starting 1 day prior to infection (9). BKIs prolonged survival and decreased the number of T. gondii brain cysts at 30 days. Considering that BKIs blocked host cell invasion and that prior experiments showed efficacy only when BKIs were given prior to infection, it could be speculated that pretreatment is required for efficacy. However, in our experiment, the oral administration of 1294 began 48 h after inoculation of 10^5 type I RH T. gondii. This method evaluates drug efficacy against an established and robust infection and demonstrates that BKIs do not need to be administered prior to infection to be effective. 1294 is highly effective against established experimental toxoplasmosis when administered orally.

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