HHS Public Access

Author manuscript

ACS Infect Dis. Author manuscript; available in PMC 2022 May 14.

Published in final edited form as:

ACS Infect Dis. 2021 May 14; 7(5): 1200–1207. doi:10.1021/acsinfecdis.0c00803.

Pyrrolopyrimidine Bumped Kinase Inhibitors for Treatment of Cryptosporidiosis

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Abstract

Bumped kinase inhibitors that target *Cryptosporidium parvum* calcium-dependent protein kinase 1 have been well established as potential drug candidates against cryptosporidiosis. Recently, BKI-1649, with a 7H-pyrrolo[2,3-d]pyrimidin-4-amine, or “pyrrolopyrimidine”, central scaffold, has shown improved efficacy in mouse models of *Cryptosporidium* at substantially reduced doses compared to previously explored analogs of the pyrazolopyrimidine scaffold. Here, two pyrrolopyrimidines with varied sunstituent groups, BKI-1812 and BKI-1814, were explored in several in vitro and in vivo models and show improvements in potency over the previously-utilized pyrazolopyrimidine bumped kinase inhibitors while maintaining equivalent results in other key properties, such as toxicity and efficacy, with their pyrazolopyrimidine isosteric counterparts.

Graphical Abstract

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Author Contributions

M.H. wrote the main manuscript. M.H., R.C., R.V., G.W., V.V., L.B., J.L., K.M., and D.K. designed and conducted the experiments. W.V.V. and D.M. assisted with experimental design. All authors reviewed and edited the manuscript. All authors have given approval to the final version of the manuscript.

ANIMAL ETHICS All animal experiments conducted at the University of Washington, USA, and AbbVie, Inc. were approved by the respective Institutional Animal Care and Use Committees at these institutions. All animals used in these studies were handled in strict accordance with practices made to minimize suffering.

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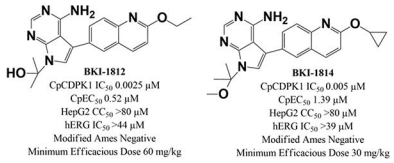
Potential Conflicts of Interest

We have no conflicts of interest to report except that W.V.V. is the president of and owns stock in ParaTheraTech Inc., a small animal health company that is developing BKIs as potential animal therapeutics. W.V.V. helped to plan the experiments and edited the paper. He did not carry out the experiments or do the initial interpretation.



Keywords

Cryptosporidium; cryptosporidiosis; bumped kinase inhibitors; pyrrolopyrimidines; pyrazolopyrimidines

Cryptosporidium has been reported to be one of the major causes of child mortality and developmental delay among several resource-limited regions throughout the world, including Sub-Saharan Africa and Southeast Asia[1–3]. In the United States, several outbreaks happen each year, including a major outbreak that infected over 400,000 in the Milwaukee, Wisconsin area in 1993[4]. Despite its prevalence, only one drug has been approved for use by the United States Food and Drug Administration (FDA). However that drug, nitazoxanide, has shown limited efficacy, fails to clear infection in immune-compromised patients, and is not approved for use in infants < 1 year-old[5–7]. Despite great progress in recent years in developing tools to advance the research and treatment of cryptosporidiosis[8–9], as well as drug development efforts that have progressed through a variety of possible drug targets and classes[10–18], a new clinical treatment has still not been found.

ATP-competitive inhibtors that target calcium-dependent protein kinase 1 (CDPK1) from Cryptosporidium parvum have shown promise as anti-parasitic agents[19]. CDPK1 is essential *for Cryptosporidium parvum* survival, but has no analogous protein in mammals, making it a promising target for treatment that may avoid toxic side effects in both humans and livestock. One class of drugs that specifically targets CDPK1 are bumped kinase inhibitors (BKIs) that inhibit cell proliferation in apicomplexan parasites by interfering with invasion and egress from mammalian host cells[20–22].

A library consisting of several hundred BKIs has been developed and tested for activity against *Cryptosporidium* in vitro and in vivo[13–14, 23–30]. Most of these compounds possess either a 1H-pyrazolo[3,4-d]pyrimidin-4-amine (pyrazolopyrimidine, PP) or a 5-aminopyrazole-4-carboxamide (AC) central scaffold (Figure 1) with substituent groups at the C3 position that occupy a, enlarged hydrophobic pocket adjacent to the glycine gatekeeper residue of CDPK1[19, 29]. Medicinal chemistry efforts around both scaffolds have yielded compounds that are potent against *Cryptosporidium* in vitro as well as in vivo mouse, piglet, and calf models[13–14, 28, 30]. However, some of these compounds have exhibited adverse side effects, including specific cardiovascular issues, such as inhibition of the human ether-à-go-go-related gene (hERG) channel that may lead to fatal arrhythmia[31–32].

Exploration of the chemistry and structure-activity relationship of a small modification to the PP scaffold, replacing nitrogen with carbon in the central scaffold, yielded a new

scaffold, 7H-pyrrolo[2,3-d]pyrimidin-4-amine (pyrrolopyrimidine, PrP) (Figure 1), for use in the development of additional BKIs[33].

One PrP, BKI-1649 (Figure 2) was shown to be efficacious in *C. parvum* (*Cp*) infected mice at significantly lower doses than many PP BKIs require[14]. BKI-1649 itself was found to be toxic at higher concentrations, as it accumulated in plasma after multiple doses and caused abortions or stillbirths in mice when dosed at therapeutic levels[27]. It therefore could not be advanced as a possible treatment candidate. Additional PrP compounds were produced and tested against *C. parvum* CDPK1 (*Cp*CDPK1) and *C. parvum* in vitro. All were shown to be equally or more efficacious in vitro than their respective PP isosteric counterparts with matching constituent groups. Two PrP BKIs-1812 and –1814 (Figure 2)[33], were selected for further testing in multiple in vitro and in vivo models to determine if safety and efficacy of these PrP BKIs would circumvent some of the shortcomings observed within the PP series of BKIs.

RESULTS

In vitro tests for 50% effective concentration (EC_{50}) against *C. parvum*, 50% inhibitory concentration (IC_{50}) against recombinant *Cp*CDPK1 protein, IC_{50} against the mammalian tyrosine kinase c-Src (a potential off-target liability in mammals), 50% cytotoxic concentration (CC_{50}) against both CRL-8155 and HepG2 cells, and solubility at pH 2.0 and 6.5 were all previously reported for these compounds (Table 1)[33].

Additional in vitro testing was conducted for both BKIs to determine their suitability for in vivo testing, including a modified Ames mutagenesis test, in vitro micronucleus genotoxicity test, hERG IC_{50}, and plasma protein binding (Table 1). All of these in vitro test results were compatible with safety in vivo. A kinome panel was also run to screen for off-target activity against human kinases (Table 2). Although BKI-1812 had an IC_{50} value of 20 nM against binding to protein kinase D3 (Prkcn, or PKD3) and an IC_{50} of 40 nM against receptor-interacting serine/threonine-protein kinase 2 (RIPK2), these are not clear safety signals that would exclude its consideration for development. In contrast, BKI-1814 had far less activity against the panel of 80 mammalian protein kinases tested.

Several pharmacokinetic (PK) parameters of BKI-1812 were previously reported for a single 25 mg/kg oral dose in adult female BALB/c mice[33]. This same assay was performed with a single 25 mg/kg oral dose of BKI-1814 to compare the two compounds. BKI-1814 had much lower oral exposure, with maximum concentration (C_{max}) and area-under-the-curve representing total exposure (AUC) values of approximately 10-fold lower and a clearance rate that was 10-fold higher than those for BKI-1812 (Figure 3).

However, the time at maximum concentration (T_{max}) and half-life (T½) values were similar for both compounds. Despite its relatively lower oral exposure, BKI-1814 was still tested in the mouse efficacy model as previous studies have suggested that in vivo efficacy of some BKI compounds is poorly predicted by high oral exposures[14]. Indeed, modeling predicts that many BKIs are delivered to cryptosporidium-infected enterocyte from the luminal surface and not from the bloodstream.

To compare the effects of BKI-1812 and BKI-1814 in vivo given their differential oral PK profiles, both compounds were tested as a dose titration in adult female interferon-γ knockout (IFN-γ KO) mice infected with nanoluciferase (Nluc) expressing *C. parvum*. Groups (n=3/dose) were given oral doses of 60 mg/kg, 30 mg/kg, and 15 mg/kg of BKIs once daily for 5 days beginning on Day 6 post-infection (PI), and infection levels were monitored up to Day 20 PI (Figure 4).

These results were compared to a negative control group dosed with vehicle only and a positive control group dosed with 100 mg/kg of BKI-1369, a PP BKI that is non-toxic in mice and has been shown to reduce infection quickly in this model[27]. The 60 mg/kg dose of BKI-1812 reduced the infection levels to below the average background signals by the fourth dose, while the 30 mg/kg and 15 mg/kg doses only partially reduced the infection levels by approximately 2 to 2.5 log units below their peaks. Both the 60 mg/kg and 30 mg/kg doses of BKI-1814 reduced the infection to below background by the fourth dose, while 15 mg/kg reduced the infection to below background after the fifth dose. Mice for all dosing groups of BKI-1812 and BKI-1814 appeared healthy and maintained their weights throughout the experiment until Day 20 PI. This was a marked improvement over the vehicle-only control mice, which rapidly lost weight, became dehydrated, and were euthanized by Day 8 PI per animal ethics protocol due to a ≥15% weight loss.

Blood was taken on the fourth day of dosing (Day 9 PI) to determine the plasma exposure after reaching steady state for each dose group (Table 3). During the analysis of BKI-1814, a second major peak was observed on the LC-MS/MS that matched the mass to charge ratio (m/z) and retention time of BKI-1649, a metabolic demethylation is likely to convert BKI-1814 to BKI-1649 (Figure 2). Due to this observation, all the plasma samples for the IFN-γ KO mouse efficacy study were reanalyzed by LC-MS/MS to determine concentrations of both the parent compound, BKI-1814, and its metabolite, BKI-1649 (Table 3).

To further evaluate the safety of these compounds in regards to effects on cardiovascular function, each was dosed in an in vivo rat cardiovascular safety pharmacology assay (three back-to-back, ascending doses of each compound, each dose continuously administered intravenously for 30 minutes). BKI-1812 showed biologically-relevant increases of 18% in heart rate and 23% in cardiac contractility at the highest dose tested, 30 mg/kg (Table 4). Plasma levels at this dose were sampled and determined by LC-MS/MS to be 188 μM. BKI-1814 showed no biologically-relevant changes to any of the measured parameters in this assay up to a maximum plasma concentration of 15.3 μM following a 30 mg/kg dose (Table 4).

DISCUSSION

In vitro results suggest that both BKI-1812 and BKI-1814 show promise for further development in the search for superior drugs against cryptosporidiosis. Both are negative in the Ames and IVMN assays and demonstrate no signs of cytotoxicity in the mammalian cells tested[33]. Both were also sufficiently soluble at pH values representative of those in the stomach and small intestine, suggesting the compounds would stay reasonably

soluble and available for absorption from the lumen and transport to the site of infection within the enterocytes[13]. Plasma protein binding is high for both of these BKIs, but it is unclear how this may relate to free levels in the fecal stream delivered to enterocytes. As *Cryptosporidium* infection is typically localized to enterocytes, the percentage of plasma binding may only be a concern in the context of toxic side effects due to systemic exposure. As such, the high degree of protein binding should be beneficial, acting as a buffer and protecting major organs in mammalian hosts from exposure-related toxicity. In the two *C. parvum* mouse efficacy experiments, it was necessary to sacrifice the vehicle control animals after Day 8 post-infection because the mice had lost >20% of their baseline body weight and appeared very ill. Thus, there were few days over which the BKI-treated animals could be compared. However, even the lower doses of BKI-1812 that did not appear to greatly reduce the parasite excretion, they prevented the weight loss and apparent illness seen in controls.

The kinome panels revealed several human kinases that are affected by both BKIs at IC_{50} values for binding of less than 10 μM, but only a small fraction that reach sub-micromolar IC_{50} values. Both showed activity against RIPK2, Prkcn, and serine/threonine-protein kinase aurora 2 (Aurora 2), with BKI-1812 also acting on mitogen-activated protein kinase kinase kinase kinase 5 (MAP4K5). RIPK2 has a biological role in promoting inflammation-related immune responses. While some drugs that inhibit RIPK2 have been investigated as a treatment for inflammation and autoimmune diseases, such as Crohn's disease, it can also potentially limit the inflammatory response to bacterial infections[34]. However, it is uncertain whether this control of inflammation may be beneficial in the case of cryptosporidiosis since gut inflammation associated with pathogenic infections has been shown to lead to environmental enteropathy (EE)[35]. EE causes sustained impairment of growth and development due to malnutrition and repeated enteric infections, which lead to damage to the gut lining and dysbiosis, even after the pathogen has been cleared[35]. Prkcn, commonly referred to as PKCn or PKD3, plays a role in multiple biological pathways, including cell cycle regulation, programmed cell death, and gene expression[36]. Aurora 2 kinase has been shown to play a role in mitosis and is essential for cell proliferation[37]. MAP4K5 expression has been studied in pancreatic cancer, with a low expression of MAP4K5 correlating with decreased survival among patients[38]. Consequently, inhibition of any one of these three kinases has the potential to increase the likelihood of oncogenesis. Therefore, extended safety studies would be required to determine if these liabilities translate to increased risk of cancer in vivo. However, a short duration of 5 days dosing needed for cryptosporidiosis therapy may alleviate some concerns about these effects.

Other concerns for BKI-1812 and BKI-1814 arise when observing their PK properties at efficacious doses. BKI-1812 reached a high C_{max} during the efficacy study, achieving 250 μM in plasma in mice dosed with 60 mg/kg. This concentration exceeds the levels that led to increases in heart rate and cardiac contractility in rats and is high enough to be of concern to inhibit mammalian kinases. Even at a dose of 15 mg/kg, plasma levels of BKI-1812 reached a C_{max} value of 50 μM, allowing for less than a four-fold difference between this treatment and levels that led to changes in cardiovascular function in rats, and it could also be of concern for inhibition of mammalian kinases. For BKI-1814, plasma levels at the efficacious doses of 30 mg/kg and 15 mg/kg remain well below acceptable levels in the rat cardiovascular assay. However, BKI-1814 is quickly metabolized into high

plasma concentrations of BKI-1649. Given BKI-1649's aforementioned abortifacient toxic side effects in pregnant mice, this leaves no room to establish a safety window between an efficacious dose of BKI-1814 and the onset of toxicity brought on by high levels of BKI-1649. It is also possible that BKI-1814 is metabolized into BKI-1649 at similar levels in the gut as are seen in plasma. Given that BKI-1649 is efficacious at such low doses[14], this could mean that the BKI-1649 that was metabolized from BKI-1814 was driving the efficacy and not the parent compound itself.

CONCLUSION

Although these two particular BKIs have properties that likely disqualify them from further consideration for human treatment, there are promising signs that the PrP scaffold may show some improvement over the more extensively explored PP BKIs in terms of potency, though this improvement is inconsistent and only results from certain pairings of constituent groups on the scaffolds. Additionally, other factors, such as hERG inhibition and cytotoxicity are equivalent between the PP and PrP isoteric counterparts when the same substituent groups are present. Mindful of these comparisons, it is worth examining both the PP and PrP scaffolds for any given set of equivalent constituent groups to determine whether the PrP variant would provide improved results. Overall, the data from these studies serve as a strong proof-of-concept for the continued development of the PrP central scaffold as inhibitors of *Cp*CDPK1 for the treatment of *Cryptosporidium*.

METHODS

Single dose pharmacokinetics.

Methods for PK analysis of mouse plasma concentrations of BKI by LC-MS/MS analysis have been previously described[14, 28, 39]. Briefly, three female BALB/c mice (10–12 weeks old) were used in each group. Each group was administered an oral dose via gavage of an individual BKI dissolved in 3% ethanol/7% Tween 80/90% saline at a dose of 25 mg/kg body weight. Blood samples were taken by tail bleeding into heparinized tubes at designated time points and centrifuged to obtain plasma. The plasma samples were frozen at −20°C.

Plasma protein binding.

Methods for in vitro plasma protein binding using dialysis membranes have been previously described[40]. Briefly, a dialysis membrane sheet (MW cutoff 3.5 kDa) (HTDialysis, LLC, Gales Ferry, CT) was soaked for 1 h in distilled water and then in 20% ethanol for 30 min. The membrane was clamped between two Teflon plates containing a row of opposing wells. Test compound in DMSO was added to 0.12 mL of serum to a concentration of 9 μM, a small aliquot was taken as a 100% recovery standard, and the solution was placed on one side of the membrane. The well on the other side of the membrane was charged with an equal volume of DPBS, and the plate was placed on an orbital shaker for 18 hours at 37°C. An aliquot was taken from each side of the membrane. One-fourth volume of acetonitrile was added to each aliquot, and the samples were centrifuged to precipitate protein. Test compound in the supernatants was quantified by LC/MS analysis to determine the concentration on each side of the membrane and the total recovery of test compound

from the device. A control dialysis was carried out with dialysis buffer on both sides of the membrane and test compound on one side to ensure that equilibration across the membrane was achieved. The fraction of compound bound to protein was calculated as bound/(unbound + bound).

Rat cardiovascular screening.

Methods for rat cardiovascular screening have been previously described[41]. Briefly, male Sprague-Dawley rats (325–375 g, Charles River, Indianapolis, IN) were anesthetized with the long-acting barbiturate Inactin (100 mg/kg i.p.). A catheter (PE50) was placed in the femoral artery for measurement of mean arterial blood pressure (MAP) and heart rate (HR). A 3F Micro-Tip catheter (Millar Instruments, Houston, TX) was advanced into the left ventricle to measure dP/dt50, a recognized index of cardiac contractility. An additional catheter (PE50) was placed in the femoral vein for administering escalating i.v. doses of drugs (dosing volume 1 mL/kg/30 min). After a 30 min baseline or equilibration period, each compound was tested using a dose-escalating protocol consisting of three 30 min escalating i.v. infusions. All studies described were acute studies, with 20% N,N-dimethylacetamide (DMA)/40% propylene glycol (PG)/40% polyethylene glycol (PEG-400) used as a formulation for acute drug administration for iv infusion at a constant rate with step-wise ascending drug concentrations encompassing therapeutic and projected supratherapeutic drug exposures. Hemodynamic data (mean arterial pressure, heart rate, and cardiac contractility) was monitored continuously, acquired every 10 s, and recorded every 5-min using a PONEMAH Physiology Platform (version number 5.2, Data Sciences International, St Paul, MN). Blood samples (each 0.25 mL volume) were obtained from the femoral artery at the end of each infusion period to assess drug exposures

C. parvum efficacy in mice.

Methods for Nluc expressing *C. parvum* in an infected IFN-γ KO mouse efficacy model have been previously described[14]. Briefly, female interferon-γ knock-out (IFN-γ KO) mice (B6.129S7-Ifngtm1Ts/J, Jackson Laboratories), aged 8–10 weeks, were infected by oral gavage (PO) with 1,000 to 10,000 UGA1 Nluc expressing C. parvum oocysts in 0.1 mL DPBS. Beginning on Day 6 PI, mice were dosed PO with BKI suspended in 0.2 mL oral vehicle (3% ethanol/7% Tween 80/90% saline) or vehicle only once daily for 5 days. Mice were moved to clean cages after each dose and fecal collection. Feces was collected daily and weighed from each group during dosing and then twice weekly out to 21 days PI. Each fecal sample was checked for luminescence[9] on day of collection and the relative luminescence unit readings were normalized to fecal sample weights.

LC-MS/MS Analysis.

All LC-MS/MS analytes were measured with an Acquity ultra-performance liquid chromatography (UPLC) system in tandem with a Xevo TQ-S micro mass spectrometer (Waters, Milford, MA, USA). PK calculations of C_{max}, T_{max}, AUC, clearance, and T½ were performed using Pharsight Phoenix WinNonlin software (Certara, St. Louis, MO).

Additional methods.

Methods for hERG channel effects assessment by automated electrophysiological patch clamp[42]. in vitro micronucleus genotoxicity assay (IVMN)[43], the 24-well modified Ames mutagenesis assay[44], and kinome profiling[45], have been previously described in detail.

ACKNOWLEDGMENTS

We thank Geno De Hostos and Robert Choy (PATH Drug Solutions), for their consultation in the drug development pathway, and Gail M. Freiberg (AbbVie) for distributing compounds and tracking them for the hERG inhibition, kinome inhibition, Ames and IVMN in vitro results and in vivo rat cardiotoxicity of the BKIs.

Funding Sources

This work was supported by the US Department of Veterans Affairs Biomedical Laboratory Research and Development Career Development Award (grant number BX002440); National Institute of Allergy and Infectious Diseases, National Institutes of Health (grant numbers R21AI123690, R01AI089441, R01AI111341, R01A1112427, and R01HD080670); and the US Department of Agriculture, National Institute of Food and Agriculture (grant number 2014-06183).

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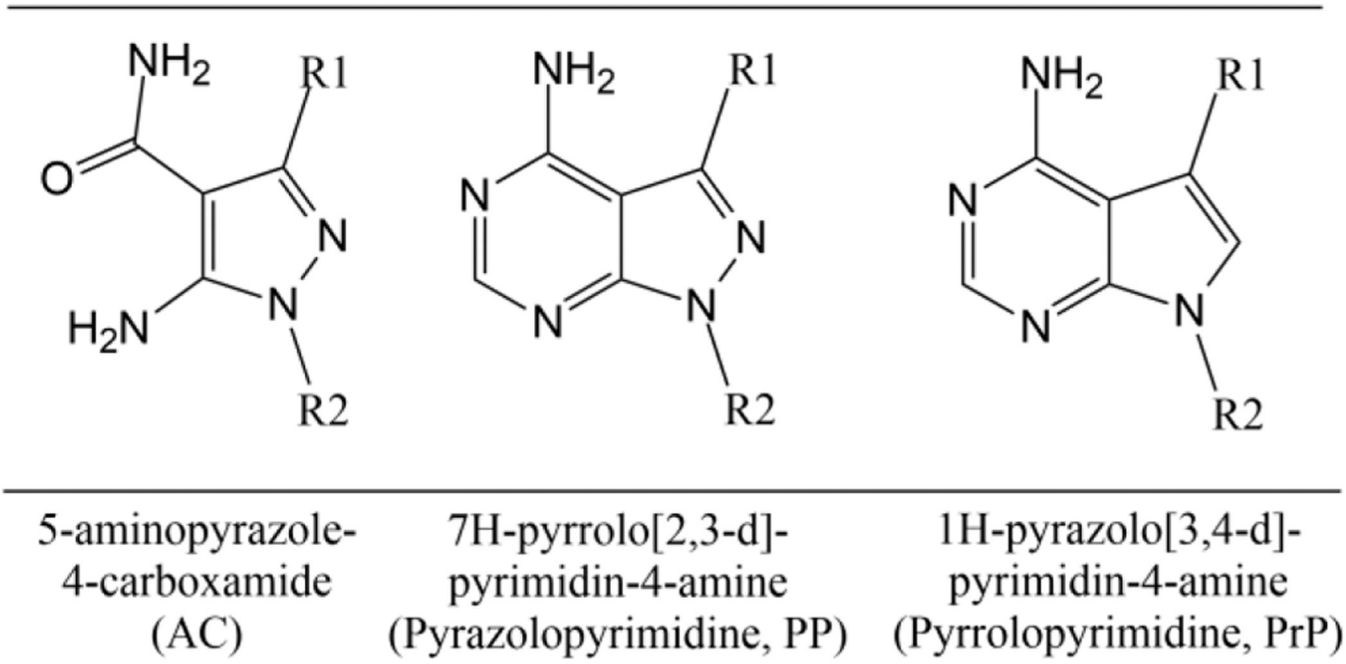


Figure 1.
Central scaffolds of bumped kinase inhibitors that target calcium-dependent protein kinase 1 in *Cryptosporidium*.

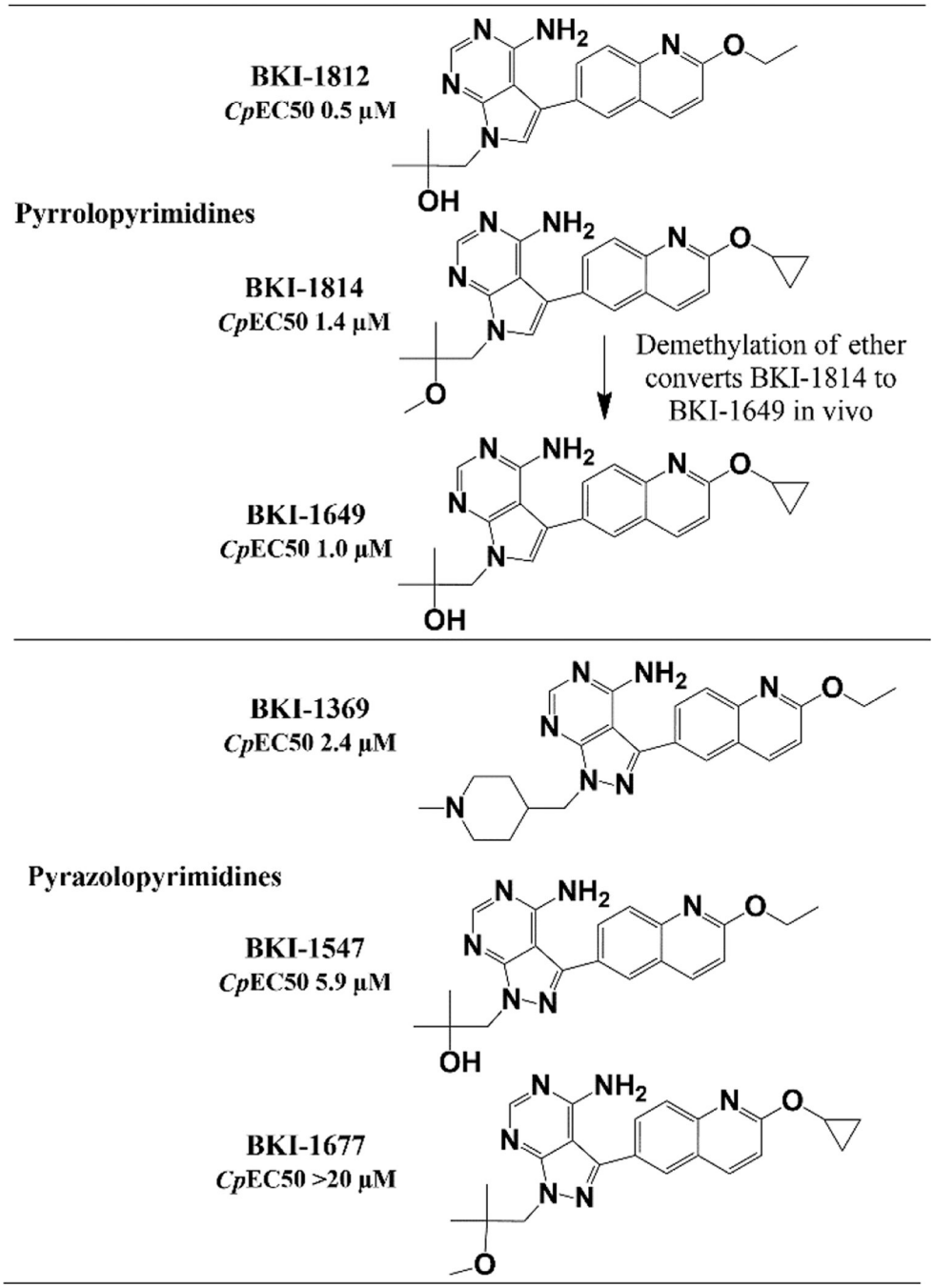
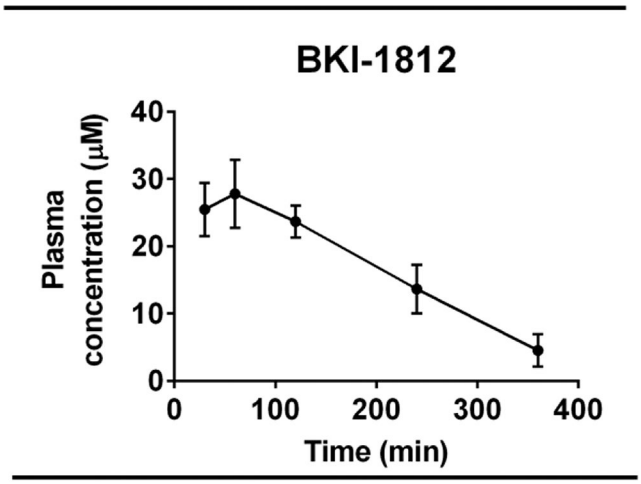
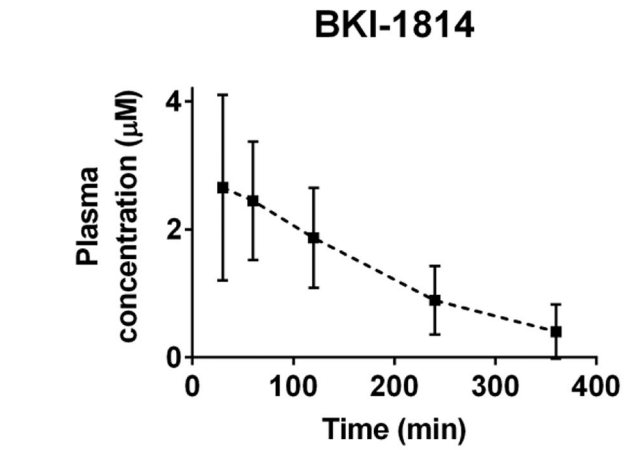


Figure 2.
Chemical structures of pyrrolopyrimidines BKIs-1812, –1814, and –1649 and pyrazolopyrimidines BKIs-1369, –1547, and –1677.





	BKI-1812	BKI-1814
Tmax (min)	50 ± 17.3	40 ± 17.3
Cmax (μM)	28 ± 5.1	2.9 ± 1.2
AUC (min*μmol/L)	8500 ± 1547	564 ± 336
T ½ (min)	99.8 ± 29.8	98.8 ± 44.5
Clearance (mL/min)	0.2 ± 0.05	2.7 ± 1.3

Figure 3.
Pharmacokinetics of a single, 25 mg/kg oral dose in uninfected adult female BALB/c mice (n=3) for BKI-1812 and BKI-1814.

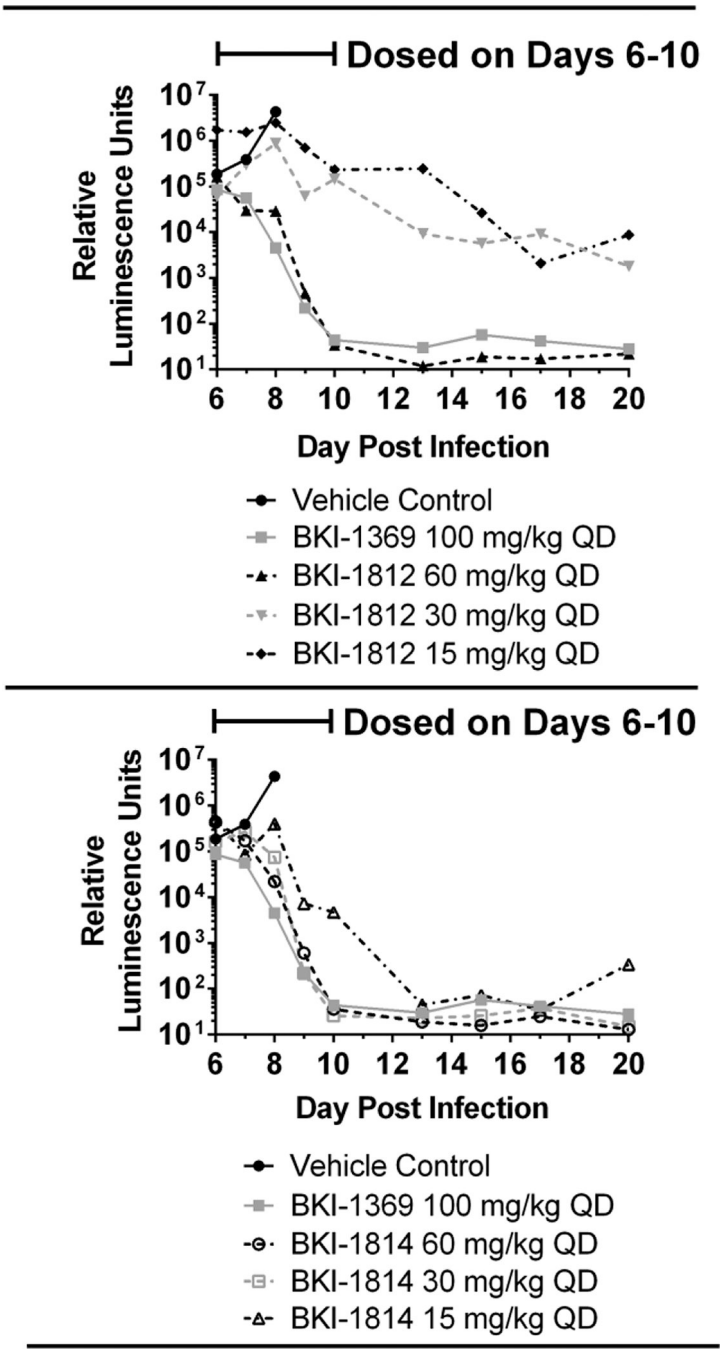


Figure 4.

Dose titration of BKI-1812 and BKI-1814 in adult female IFN-γ KO mice (n=3) infected with nanoluciferase-expressing *Cryptosporidium parvum*. Blood was sampled on Day 9 post infection for LC-MS/MS analysis of plasma concentrations, as indicated in Table 3.

Table 1.

In vitro properties of pyrrolopyrimidines, BKI-1812, BKI-1814, and BKI-1649.

	BKI-1812	BKI-1814	BKI-1649
***Cp*CDPK1 IC_{50} (μM)**	0.0025	0.005	0.0022
***C. parvum* EC_{50} (μM)**	0.52	1.39	1.03
CRL-8155 CC_{50} (μM)	>80	>80	>40
HepG2 CC_{50} (μM)	>80	>80	>40
Solubility pH 2.0 (μM)	>100	>100	>100
Solubility pH 6.5 (μM)	96.5	>100	83
hERG IC_{50} (μM)	>44	>39	>30
Modified Ames	Negative	Negative	ND*
In vitro mononucleus genotoxicity	Negative	Negative	ND*
Plasma Protein Binding Mouse	85.0%	99.9%	94%
Plasma Protein Binding Rat	91.5%	94.7%	90%
Plasma Protein Binding Human	99.9%	99.9%	56%

ND = not done

Table 2.

In vitro inhibition of human kinases.

	BKI-1812	BKI-1814
Kinase	**IC_{50} (μM)**	
Prkcn	0.0198	0.326
RIPK2	0.0375	0.203
Aurora2	0.284	0.405
MAP4K5	0.584	>10
SIK1	1.01	4.82
Blk	1.11	>10
DDR1	1.38	>10
Lck	1.48	4.2
Flt1	2.07	6.68
Aurora1	2.33	2.91
IRAK4	2.54	>10
MEK1	2.69	3.5
MAP4K3	2.83	>10
MST1	2.94	4.62
CDK8/Cyclin C	3.7	>10
TNK2	3.71	>10
RET	4.02	5.18
MINK1	4.3	>10
FGR	4.39	>10
MAP4K1	4.42	>10
MAP4K2	4.59	8.16
MAP4K4	4.79	>10
CSF1R	5.14	6.11
ErbB2	6.6	>10
HCK	7.04	>10
STK33	7.96	>10
CSK	8.75	>10
TYRO3	>10	6.32
All others tested	>10	>10

Table 3.

Mean ± standard deviation plasma concentrations of BKIs –1812, –1814, and –1649. Taken from the 4th day (day 9 PI) of dosing *C. parvum* infected adult female IFN-γ KO mice.

BKI-1812	**60 mg/kg**	**30 mg/kg**	**15 mg/kg**
Time sampled	**Plasma concentration (μM)**		
predose	15.7 ± 11.7	2.1 ± 0.2	1.5 ± 0.3
1 h post dose	258.9 ± 22.5	111.4 ± 71	49.4 ± 26.3
2 h post dose	250.5 ± 48.4	115.7 ± 65.9	37.4 ± 19.3
4 h post dose	170.1 ± 110.8	54.6 ± 31.7	14.1 ± 9.2
BKI-1814	**60 mg/kg**	**30 mg/kg**	**15 mg/kg**
Time sampled	**Plasma concentration (μM)**		
predose	1.83 ± 1.4	0.03 ± 0.06	0.24 ± 0.2
0.5 h post dose	17.5 ± 10.7	7.1 ± 2.5	1.68 ± 0.6
1 h post dose	19.5 ± 9.8	6.88 ± 1.5	2.46 ± 0.5
2 h post dose	18.2 ± 10.2	5.86 ± 1.6	2.25 ± 0.8
BKI-1649*	**60 mg/kg**	**30 mg/kg**	**15 mg/kg**
Time sampled	**Plasma concentration (μM)**		
predose	346 ± 78	113 ± 31	76 ± 1.2
0.5 h post dose	340 ± 177	110 ± 65	79 ± 44
1 h post dose	345 ± 188	111 ± 66	82 ± 46
2 h post dose	360 ± 199	134 ± 81	93 ± 51

*BKI-1649 formed in vivo by demethylation of ether on BKI-1814

Table 4.

Cardiovascular effects of intravenous infusion in anesthetized rats (n=3). A change of ± 15% is considered biologically relevant for mean arterial pressure (MAP) or heart rate (HR). A change of ± 20% is considered biologically relevant for cardiac contractility (dP/dt@50). Plasma concentration values are listed as mean ± SEM. % values are listed as mean change relative to vehicle.

	Dose (mg/kg)	Plasma Concentration (μM)	MAP (%)	HR (%)	dP/dt@50 (%)
BKI-1812	3	1.59 ± 0.42	−2	5	5
	10	11.3 ± 0.5	4	13	10
	30	188 ± 44.1	−5	18	23
BKI-1814	3	1.74 ± 0.04	−3	−3	4
	10	6.67 ± 0.37	−4	−5	6
	30	15.3 ± 0.04	−6	−9	16