Bumped-Kinase Inhibitors for Cryptosporidiosis Therapy

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Bumped kinase inhibitors (BKIs) of *Cryptosporidium parvum* calcium-dependent protein kinase 1 (*Cp*CDPK1) are leading candidates for treatment of cryptosporidiosis-associated diarrhea. Potential cardiotoxicity related to anti–human ether-à-go-go potassium channel (hERG) activity of the first-generation anti-*Cryptosporidium* BKIs triggered further testing for efficacy. A luminescence assay adapted for high-throughput screening was used to measure inhibitory activities of BKIs against *C. parvum* in vitro. Furthermore, neonatal and interferon γ knockout mouse models of *C. parvum* infection identified BKIs with in vivo activity. Additional iterative experiments for optimum dosing and selecting BKIs with minimum levels of hERG activity and frequencies of other safety liabilities included those that investigated mammalian cell cytotoxicity, *C. parvum* proliferation inhibition in vitro, anti–human Src inhibition, hERG activity, in vivo pharmacokinetic data, and efficacy in other mouse models. Findings of this study suggest that fecal concentrations greater than parasite inhibitory concentrations correlate best with effective therapy in the mouse model of cryptosporidiosis, but a more refined model for efficacy is needed.

Keywords. Cryptosporidiosis treatment; calcium-dependent protein kinase 1; bumped kinase inhibitors.

Cryptosporidiosis is a significant contributor to global diarrheal morbidity and mortality. *Cryptosporidium* is associated with moderate-to-severe diarrhea, stunting, and death in children <2 years old in resource-limited environments [1–3]. In immunocompromised patients, cryptosporidiosis diarrhea may be life threatening.

Cryptosporidium organisms are obligate intracellular parasites that live in intracellular but extracytosolic parasitophorous vacuoles located beneath the apical plasma membrane of infected intestinal epithelial cells [4]. Large waterborne outbreaks are common, such as the 1993 Milwaukee, Wisconsin, incident in which 403 000 cases were reported after the local water supply became contaminated with *Cryptosporidium* oocysts [5]. The infectious dose for humans ranges from 9 to 132 oocysts, depending on the strain of *Cryptosporidium* [6, 7]. The incapacitating nature of the diarrhea, high rate of infection, low infectious dose, association with death and stunting, and resistance of parasite to elimination from the environment are all factors that have heightened public health concerns about the disease.

The only Food and Drug Administration–approved therapy for cryptosporidiosis, nitazoxanide, was shown in a randomized trial to reduce the duration of diarrhea by approximately 2 days in immunocompetent individuals [8]. Nitazoxanide also has limited efficacy and a high failure rate in immunocompromised individuals [9]. It is not approved for use in children <12 months of age, whereas studies in malnourished children show poor response rate [10]. With the burden of disease falling disproportionately on young children in regions with a high human immunodeficiency virus prevalence [11] and widespread malnutrition, better drugs are needed for cryptosporidiosis therapy.

Multiple studies have shown that inhibition of apicomplexan calcium-dependent protein kinases (CDPKs) interfere with the parasites' ability to invade or egress mammalian host cells [12–14]. Calcium regulation in diverse metabolic and functional pathways is essential to apicomplexan parasites' survival [15–18]. It has previously been demonstrated that *C. parvum* growth was inhibited by BKIs that target *Cp*CDPK1 [19]. BKI 1294 exhibited anti-*Cryptosporidium* efficacy in the SCID/beige immunocompromised mouse and the newborn calf *C. parvum* clinical models [20, 21]. Since *Cryptosporidium* infections are predominant in the gastrointestinal tract, therapeutic agents with unconventional pharmacokinetic and pharmacodynamic properties may be required for effective treatment. Here we report our progress to identify BKIs that are effective against *Cryptosporidium*.

MATERIALS AND METHODS

Methods for the propagation of *C. parvum* (IOWA-II strain) in calves [22, 23], creation of a transgenic *C. parvum* strain

UGA1 transfected to express nanoluciferase (Nluc) [24], purification of oocysts from fecal specimens [23, 25], synthesis of BKIs [19, 26, 27], measurement of in vitro CpCDPK1 activity [19], pharmacokinetic analysis of plasma and fecal BKI concentrations [15, 21], in vitro investigation of BKI plasma protein binding [28], and determination of BKI efficacy in neonatal mice [21] and adult SCID/beige mice [20] have been previously described. Variances from published procedures are included in the Supplementary Materials and Methods.

C. parvum Growth Inhibition by BKIs

Assays to determine growth inhibition and the 50% effective concentration (EC_{50}) were performed using a *C. parvum* strain UGA1 expressing Nluc in human ileocecal adenocarcinoma (HCT-8) cells (ATCC, Manassas, VA) as previously described [24] with the following modifications. HCT-8 cells were inoculated onto 96-well plates and allowed to grow for 48 hours to 90%–100% confluence. *C. parvum* oocysts were incubated for 10 minutes in 10% bleach at room temperature and washed with Dulbecco's phosphate-buffered saline. One thousand oocysts per well were applied to plates with Roswell Park Memorial Institute 1640 (RPMI) medium supplemented with 10% horse serum and 1% penicillin/streptomycin at the same time as compound addition and incubated for 48 hours. The monolayers were lysed for 1 hour before Nano-Glo luciferase reagent (Promega, Madison, WI) was added, and relative luminescence units (RLU) were determined on an EnVision Plate Reader (Perkin Elmer, Waltham, MA). EC_{50} values were calculated using Prism (GraphPad, La Jolla, CA). Assays were repeated 2–3 times, and representative data are expressed as means ± standard deviation.

Efficacy in Adult Interferon γ (IFN-γ) Knockout (KO) Mice

Female IFN-γ KO mice (B6.129S7-Ifngtm1Ts/J, Jackson Laboratories) aged 8–10 weeks were infected by oral gavage with 1000–10 000 *C. parvum* UGA1 oocysts carrying Nluc in 0.1 mL of Dulbecco's phosphate-buffered saline. Beginning on day 3 or 6 after infection, mice were dosed orally with BKI suspended in 0.2 mL of 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. Fecal specimens were collected daily and weighed from each group during dosing and twice weekly out to 20–21 days after infection. Each fecal sample was checked for luminescence on the day of collection [24], and RLU readings were normalized to fecal sample weights. For subcutaneous doses, the vehicle was 60% Phosal 53 MCT/30% PEG/10% ethanol, as oral vehicle can cause inflammation when injected (unpublished data). GraphPad Prism was used to determine the statistical significance of outcomes, using the Student *t* test and the correlation coefficient (8) for efficacy, with respect to systemic exposure (as measured by the area under curve [AUC]) and fecal concentrations.

Animal Ethics Statement

All animal experiments were approved by the institutional animal care and use committees at the participating universities (University of Washington, University of Georgia, University of Arizona, and University of Texas Medical Branch).

RESULTS

Lead BKIs were selected as potential *Cryptosporidium* therapies on the basis of favorable in vitro parameters, including c-Src off-target effects, mammalian cytotoxicity, hERG activity, and efficacy in the HCT-8 infection model (Table 1 and Figure 1A). Select lead candidates were evaluated for efficacy in neonatal mice and in plasma and fecal specimens from adult mice after oral dosage. BKIs were also eliminated on the basis of efficacy or exposure findings that approached or exceeded hERG or toxicity levels, as indicated by the in vitro results (Tables 1 and 2 and Figure 1A). Adult mouse efficacies were evaluated for all remaining BKIs, with analyses of efficacious compounds repeated to determine their effect at different treatment times, concentrations, and dose administrations (Figures 1–3).

Enzymatic and Cellular Potency and Off-Target Cytotoxicity

The BKIs described here were tested for the inhibition of recombinant CpCDPK1 activity (Table 1). All BKIs were counter-screened against the mammalian tyrosine kinase c-Src and screened for mammalian cytotoxicity in vitro to eliminate toxic compounds. BKIs with activity against c-Src were eliminated because of concerns about off-target inhibition of mammalian kinases [29]. BKI 1294 had previously been reported to inhibit hERG at concentrations needed for efficacy, and hERG is a cardiotoxicity liability to be avoided [27, 30]. Accordingly, the remaining BKIs were screened for hERG inhibition by the thallium-flux assay for potassium channels [31, 32]. The BKIs listed in (Table 1) have a ≥10 fold higher hERG 50% inhibitory concentration (IC_{50}) than BKI 1294 and thus have less potential for cardiotoxicity.

In Vitro Inhibition of *Cryptosporidium* Growth by BKIs

Nluc-expressing *C. parvum* oocysts were used to infect HCT-8 cells to assay for cellular BKI inhibition [24]. Compounds with an IC_{50} of >0.10 μM against recombinant CpCDPK1 enzyme showed little or no activity against the parasites in vitro, whereas compounds with a CpCDPK1 IC_{50} of <0.020 μM inhibited *Cryptosporidium* proliferation in the low micromolar range (Table 1). BKIs RM-1-22, RM-1-89, 1243, 1266, 1416, 1610, and 1624 were all eliminated from further examination because of poor activity, off-target c-Src inhibition, or mammalian cell cytotoxicity (Figure 1B).

BKI Efficacy Against *C. parvum* Infection in Neonatal Mice

Twelve BKIs, 1294, 1318, 1369, 1412, 1534, 1546, 1550, 1553, 1556, 1557, 1649, and 1663, have good *C. parvum* growth inhibition and little cytotoxicity or c-Src inhibition (Table 1). These

Table 1. In Vitro Properties and Neonatal Mouse Efficacy of Bumped-Kinase Inhibitors

Compound	R1	R2	IC$_{50}$, μM			C. parvum EC$_{50}$, μM, Mean ± SD	CC$_{50}$, μM			Percentage of Protein Bound in Plasma, by Species		Percentage Reduction in Parasite Burden in Neonatal Mice, Mean ± SD
			CpCDPK1	c-SrC	hERG		HepG2	CRL-8155	HCT-8	Human	Mouse	
RM-1–22	(structure)	(structure)	0.059	0.121	ND	5.3 ± 0.3	30.9	10.4	>20	ND	ND	ND
RM-1–89	(structure)	(structure)	0.012	0.549	>10	0.7 ± 0.2	ND	30	>20	ND	ND	ND
1243	(structure)	(structure)	>5	>10	ND	>20	ND	ND	>20	ND	ND	ND
1266	(structure)	(structure)	0.58	>10	ND	>80	>40	ND	>20	ND	ND	ND
1294	(structure)	(structure)	0.001	>10	0.76	2.7 ± 1	>40	>40	>20	85	84	56 ± 14
1318	(structure)	(structure)	0.001	>10	10	3.2 ± 0.5	>40	>40	>20	77	55	69 ± 11
1369	(structure)	(structure)	0.001	>10	10	2.4 ± 0.7	>40	>40	>20	77	37	74 ± 10
1412	(structure)	(structure)	0.008	>10	>10	2.2 ± 0.6	>40	>40	>20	94	85	14 ± 15
1416	(structure)	(structure)	0.289	>10	ND	20 ± 2.8	ND	ND	>20	ND	ND	ND
1534	(structure)	(structure)	0.005	>10	25	2.2 ± 0.3	>40	39.9	>20	99.9	99.9	61 ± 13
1546	(structure)	(structure)	0.001	>10	>30	1.3 ± 0.3	>40	>40	>20	92	81	33 ± 8
1550	(structure)	(structure)	0.001	7.4	>30	5.5 ± 2.4	>40	>40	>20	83	84	50 ± 14
1553	(structure)	(structure)	0.002	>10	>30	1.6 ± 0.3	>40	39.5	>20	91	90	36 ± 12
1556	(structure)	(structure)	0.003	>10	25	3.2 ± 0.6	>40	22.7	>20	99.9	86	61 ± 11
1557	(structure)	(structure)	0.007	>10	14	5.5 ± 0.3	>40	31.1	>20	91	84	33 ± 11
1610	(structure)	(structure)	0.462	ND	ND	37.7 ± 5.4	>40	>40	>20	ND	ND	ND
1624	(structure)	(structure)	0.426	ND	ND	>80	>40	>40	>20	ND	ND	ND
1649[a]	(structure)	(structure)	0.002	>10	>30	2.3 ± 1.3	>40	>40	>20	56	90	56 ± 10
1663	(structure)	(structure)	0.021	>10	7.6	6.7 ± 0.5	>40	>40	>20	96	86	76 ± 13
Paromomycin[b]	…	…	…	…	…	…	…	…	…	…	…	18 ± 14

The central scaffold of each compounds is , unless otherwise indicated.

Abbreviations: CC$_{50}$, 50% cytotoxicity concentration; c-SrC, ; CpCDPK1, Cryptosporidium parvum ; EC$_{50}$, 50% effective concentration; IC$_{50}$, 50% inhibitory concentration; ND, not determined.

[a]The central scaffold is .

[b]Paromomycin was selected as the standard comparator because it consistently yielded better activity than nitazoxanide in experimental mouse models.
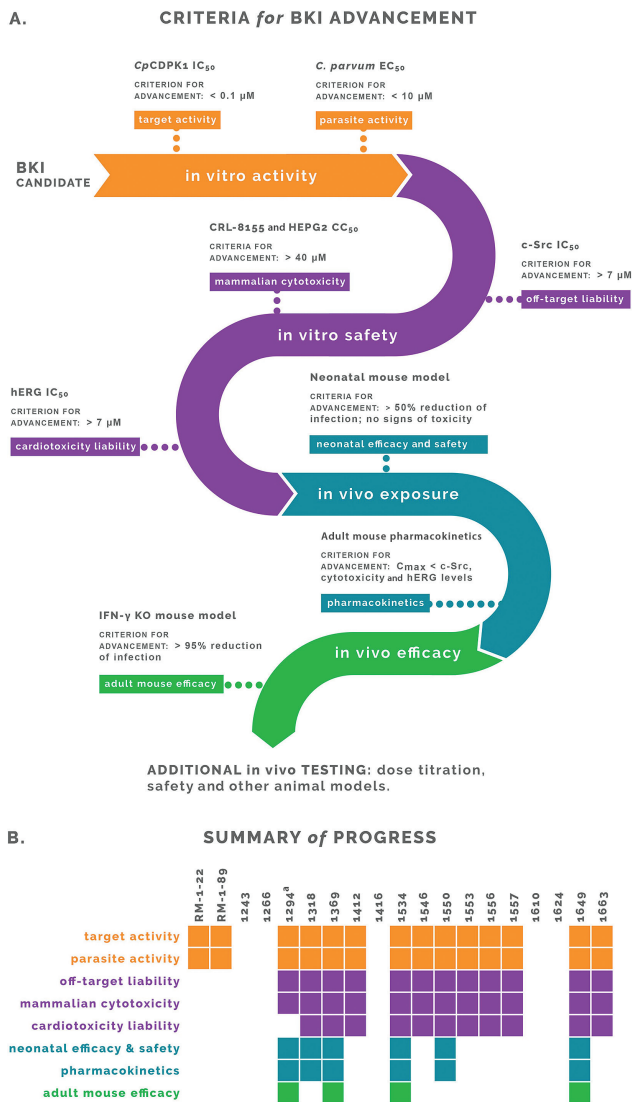
Figure 1. Criteria for continuing or ceasing additional investigation of bumped-kinase inhibitor (BKI). *A,* The colors reflect distinct stages of evaluating BKIs for their suitability as a therapy for cryptosporidiosis. Criteria for advancement (ie, continuing additional investigation) are listed for each assay. *B,* Summary of the progression of each individual BKI. Colored blocks are filled in when the BKI satisfies the criteria for advancement and left blank when the BKI fails to meet the criteria and was eliminated from additional investigation. Abbreviations: CC_{50}, 50% cytotoxicity concentration; C_{max}, maximum concentration; *Cp*CDPK1, *Cryptosporidium parvum* calcium-dependent protein kinase 1; *C. parvum, Cryptosporidium parvum;* EC_{50}, 50% effective concentration; IC_{50}, 50% inhibitory concentration; IFN-γ, interferon γ; KO, knockout. [a]Included as a control.

BKIs were tested in a neonatal mouse model of *C. parvum* infection at an oral dose of 25 mg/kg given twice daily. For all compounds, infection levels decreased by 14%–76% relative to levels after vehicle treatment (Table 1). The least effective BKI in this series, 1412, reduced oocyst shedding by a level similar to that for paromomycin (Table 1). BKIs 1556, 1649, and 1663 showed signs of toxicity—specifically, protruding sternums—in infected neonatal mice. Although it is not intended to evaluate toxicity, the neonatal mouse model is practical for investigation

and interpretation of data for various aspects of cryptosporidiosis in human infants, including potential harm from drug treatment. For this reason and its hERG-associated liability (Table 1), 1663 was eliminated from further consideration (Figure 1B). Compounds 1412, 1546, 1553, and 1557 were eliminated because of suboptimal efficacy in neonatal mice, defined as a <50% reduction of infection levels (Figure 1B).

BKI Levels in Mouse Plasma and Feces

Plasma and stool drug levels were determined after oral dosing in adult mice (Table 2). BKI 1556 was eliminated because its high oral exposure led to a concern that it would reach hERG-inhibiting plasma concentrations (Table 1 and Figure 1B). BKI 1649 was further evaluated at doses of <25 mg/kg twice daily, because 25 mg/kg twice daily was toxic in infected neonatal mice.

BKI Efficacy in the IFN-γ KO Mouse Model of *C. parvum* Infection

It is not yet clear which mouse model of *Cryptosporidium* infection predicts effective therapy in humans. In the IFN-γ KO mice model, infection is progressive, whereas immunocompetent neonatal mice are transiently infected for 10 days before the infection is cleared [24]. BKIs 1266, 1294, 1318, 1369, 1534, 1550, and 1649 were delivered orally for 5 days beginning at day 3 after infection (Figure 2A and 2B). Compound 1266 (*Cp*CDPK1 IC_{50}, 0.58 µM; *C. parvum* EC_{50}, >80 µM) was included as a negative control and showed no clearance of *C. parvum*, compared with untreated controls (Figure 2A). In contrast, BKIs 1294, 1369, and 1534 significantly reduced parasite shedding (*P* < .002; Figure 2A and 2B). BKIs 1550 and 1318 (*P* < .5 for both) only slightly reduced the parasite load and were eliminated from further consideration (Figure 2B). BKI 1649 was delivered at a dose of 6 mg/kg/day, owing to its high oral exposure (Table 2) and neonatal mouse toxicity at 25 mg/kg twice daily, and still significantly reduced infection (*P* < .01) and was therefore worthy of further evaluation.

No clinical signs of illness were observed in the infected IFN-γ KO mice, except for gradual weight loss and decreased activity in control and unsuccessfully treated mice. Infection intensities varied between the different IFN-γ KO mouse experiments. This can be observed by higher fecal luciferase levels in the second experiment (Figure 2B). The control mice referenced in (Figure 2B) lost ≥20% of their baseline body weight—the threshold at which euthanasia is required—by 20 days after infection, unlike the control mice referenced in (Figure 2A), where this threshold was not reached by the end of the experiment. The increased infection level was associated with relapsed oocyst shedding after the completion of dosing in some experiments. BKI 1294 administration consistently led to a significant reduction of infection, but a recurrent increase in parasite excretion was observed (Figure 2B). This was different than the initial experiment, in which no recurrent increase was noted with the same dose (Figure 2A). The greater relapse in the

Table 2. Pharmacokinetic Data of Bumped-Kinase Inhibitors (BKIs) in Uninfected Adult Mice After Oral Dosing

BKI Compound, by Dosing Route	Dose, mg/kg	C_{max}, µM	T_{max}, min	AUC, µmol/L min	Half-life, min	Oral Clearance Rate, mL/min	Fecal Concentration, µM
Oral[a]							
1294	10	0.75	120	430	205	1.69	4.4
1318	10	6.5	120	2209	909	0.16	0.44
1369	10	0.26	200	47	197	0.78	5.20
1412	10	0	0	0	...[b]	...[b]	1.83
1534	10	0.23	30	58	66	10.5	10.1
1546	10	0	0	0	...[b]	...[b]	5.29
1550	10	3.9	30	309	41	2.1	0.036
1553	10	12.8	320	13725	1188	0.02	0.52
1556	25	7.0	50	2082	320	0.56	...
1557	10	0.88	30	67.4	49	10.9	1.67
1649	10	4.9	400	6416	895	0.01	5.76
Subcutaneous[c]							
1294	60	12.7	280	12249	6066	...	0.92
1534	60	4.2	180	3223	1831	...	0.44

Abbreviations: AUC, area under the curve; C_{max}, maximum concentration; T_{max}, time at which maximum concentration was observed.

[a] 25 mg/kg dose.

[b] No systemic exposure detected.

[c] 60 mg/kg dose.
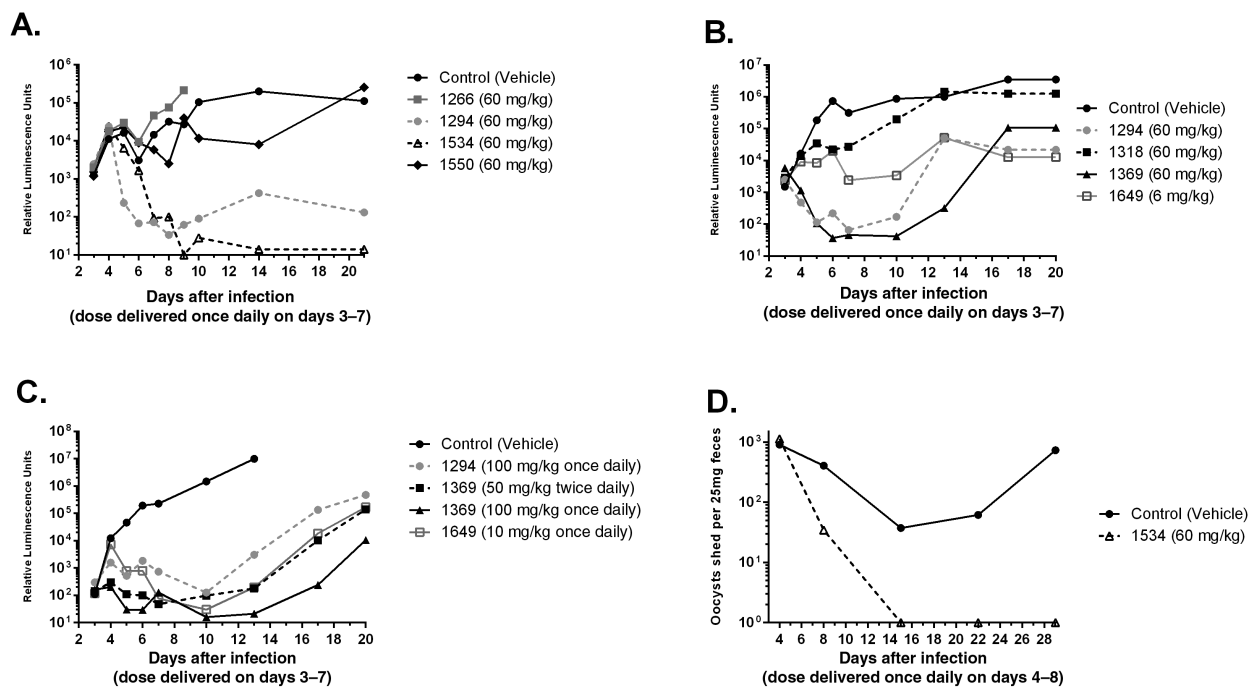
Figure 2. Efficacy of bumped-kinase inhibitor (BKIs) in mouse models of *Cryptosporidium parvum* infection. *A–C*, Efficacies against *C. parvum* excretion in adult interferon γ knockout mice, based on nanoluciferase activity log RLU plotted on y-axis (*A–C*), and efficacy of BKI 1534 in SCID/beige mice, using reverse transcription–polymerase chain reaction to quantify infection (*8*). *A*, BKIs were delivered orally for 5 days, beginning 3 days after infection. In vivo inhibition by BKIs 1266, 1294, 1534, and 1550. BKI 1266, with limited inhibition of the *C. parvum* calcium-dependent protein kinase 1 target enzyme, was included as a negative control. BKIs 1294 and 1534 significantly reduced parasite shedding as compared to controls. The *C. parvum* infectious dose was 10 000 oocysts. *B* and *C*, Significant initial reduction of parasite burden by BKIs, even in the case of higher fecal luciferase levels, which reflect higher parasites load. Parasite excretion rebounds when control animals show a higher infectious burden (*8*). BKIs 1294 (100 mg/kg once daily), 1369 (100 mg/kg once daily or 50 mg/kg twice daily), and 1649 (10 mg/kg once daily) efficacies (uninfected control mice had the highest recorded levels; *C*). BKIs substantially reduced *Cryptosporidium* proliferation, as shown by lower oocyst counts. A once-daily BKI 1369 dose of 100 mg/kg significantly reduced the infection burden (by 100 000-fold), relative to that in untreated controls. BKI 1649 was administered at lower doses than other compounds owing to its high oral exposure, but it still significantly reduced infection relative to controls. The *C. parvum* infectious dose was 1000 oocysts. *D*, BKI 1534 delivered orally at a dose of 60 mg/kg for 5 days cleared infection with no recurrence. Assays were performed at the University of Georgia (Athens; *A*), the University of Washington (Seattle; *B* and *C*), and the University of Texas Medical Branch (Galveston; *D*). For assays with results in panels *A* and *B*, the percentage inhibition of infection, relative to that in controls, after treatment (day 10) was calculated for each compound as follows: 1294, 99.5%; 1534, 99.4%; and 1550, 59.7% (*8*); and 1294, 99.9%; 1318, 37.6%; 1369, 99.9%; and 1649, 98.9% (*8*). There were 3 mice in each experimental group.

second experiment as compared to the first may have been due to the increased infection level, judging by the more rapid onset of infection and higher levels of luciferase signal. A recurrent rise in infection level was also observed after BKI 1369 treatment (Figure 2B), again perhaps due to the increased infection level. However, the level of infection after treatment remained at <15% of that for controls, and the mice appeared healthy for the duration of the experiment.

The efficacies of BKIs 1294, 1369, and 1649 were tested at higher oral doses (Figure 2C). BKI 1369 was administered at 100
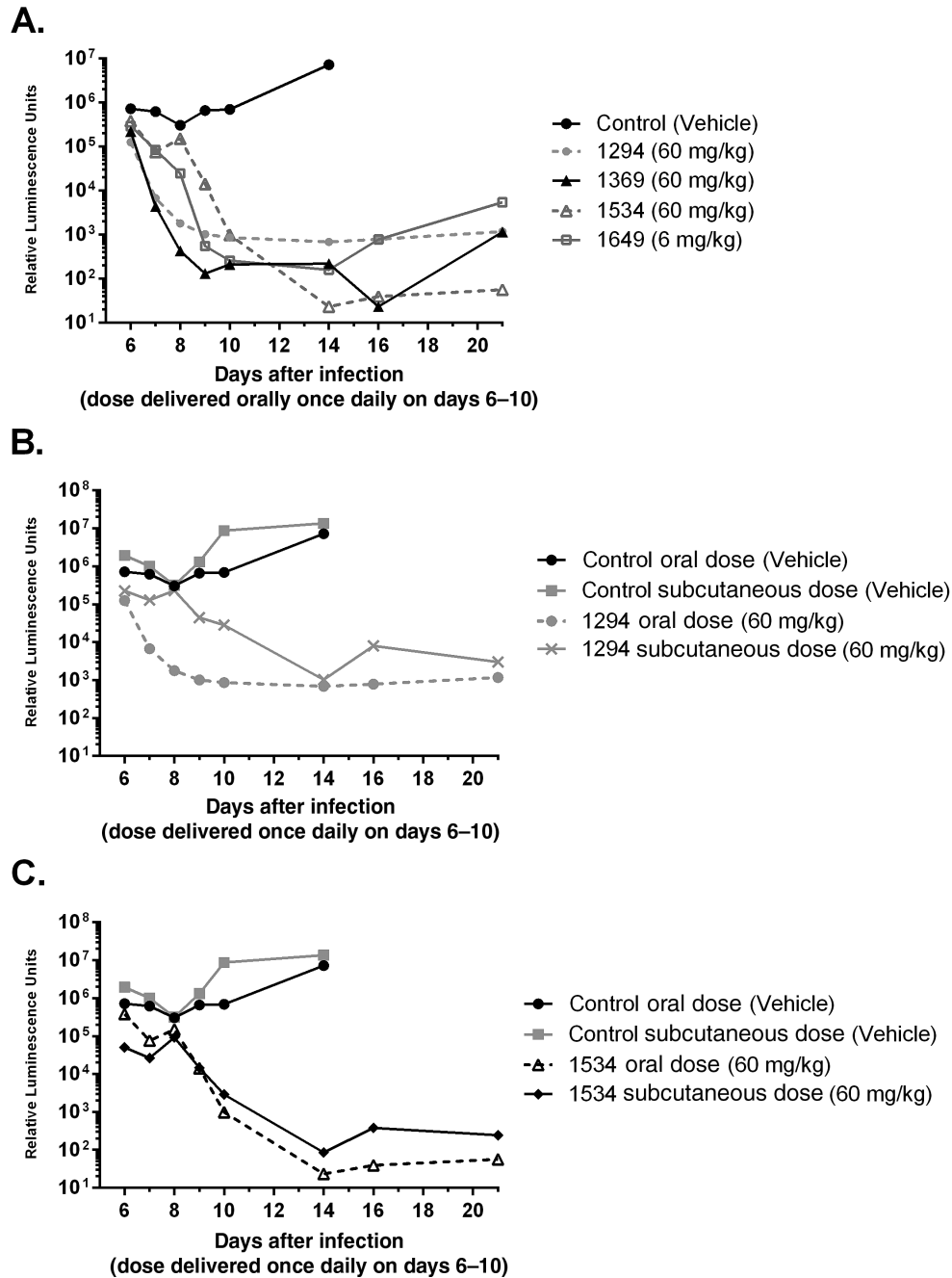
Figure 3. Efficacy in adult interferon γ knockout mice with dosing starting on day 6 after infection. In all mice, the nanoluciferase-expressing *Cryptosporidium parvum* infectious dose was 1000 oocysts. *A*, Efficacy plots of bumped-kinase inhibitors (BKIs) 1294, 1369, 1534, and 1649 used for treatment of highly established infection when administered orally at 60 mg/kg once daily for 5 days. BKI 1649 was administered at 6 mg/kg once daily. All BKIs showed significant decreases in infection. *B*, Efficacy of subcutaneous versus oral dosing of mice with 1294 at 60 mg/kg, starting on day 6 after infection, for 5 days. BKI 1294 showed a significantly greater reduction in oocyst excretion when delivered orally as compared to an equivalent dose delivered subcutaneously ($P < .05$). This is probably due to a fecal concentration of orally administered 1294 that was higher than the 50% effective concentration (EC_{50}) early in the treatment period and to the longer presence of the orally administered 1294 in the lumen relative to the subcutaneously administered dose, for which the level after a single dose was below the EC_{50}. *C*, Efficacy of subcutaneous versus oral dosing with 1534 at 60 mg/kg, starting on day 6 after infection, for 5 days. No significant difference was seen in parasite excretion between the groups receiving the BKI doses.

Table 3. Single-Dose Fecal Concentrations of Bumped-Kinase Inhibitors 1294 and 1534 in Uninfected Adult Mice, Compared With the Fifth-Dose Fecal Concentrations in Infected Adult Mice

	Concentration, μM	
Infection Status, Dose	1294	1534
Uninfected, single dose[a]		
25 mg/kg orally	4.4	10.1
60 mg/kg subcutaneously	0.92	0.44
Infected, fifth dose[b]		
60 mg/kg orally	4.1	17.8
60 mg/kg subcutaneously	1.1	4.7

[a]Concentration (weight/volume) in pooled fecal specimens from adult uninfected mice given a single oral or subcutaneous dose.

[b]Concentration (weight/volume) in pooled fecal specimens from interferon γ knockout mice infected with *Cryptosporidium parvum* after the fifth oral or subcutaneous dose.

mg/kg orally once daily or at 50 mg/kg orally twice daily, and BKI 1649 was delivered at 10 mg/kg orally once daily. Compound 1294 was included at 100 mg/kg orally once daily. The infection in this experiment was even more robust, with parasite loads in control mice rising to their highest recorded levels. The control mice had weight loss requiring euthanasia at day 13 after infection, a week earlier than the experiment described in Figure 2B. This infection intensity probably led to BKI 1294 having less apparent efficacy (Figure 2C) than in previous experiments (Figure 2A and 2B), while still significantly reducing infection ($P < .05$). Nonetheless, all BKIs had a substantial effect on infection (Figure 2C). BKI 1369 at 100 mg/kg once daily significantly reduced the infection ($P < .05$) to levels >100 000 times lower than those in controls. BKI 1369, when administered at 50 mg/kg twice daily, considerably reduced infection levels ($P < .05$) but with a faster recurrence and greater total oocyst excretion, compared with the same total dose given once daily. BKI 1649 also significantly reduced infection levels ($P < .05$) at a dose of 10 mg/kg once daily.

SCID/Beige Mouse *C. parvum* Model

BKI 1534 was tested in the SCID/beige mouse *C. parvum* infection model. BKI 1534 given orally at 60 mg/kg for 5 days cleared the infection, with no recurrence (Figure 2D).

BKI Therapy of Highly Established Infection in the IFN-γ KO Mouse *C. parvum* Model

To test the effects of BKIs on higher infection levels, we evaluated the efficacy of our lead inhibitors by delaying dosing to day 6 after infection in IFN-γ KO mice. BKIs 1294, 1369, and 1534 were administered orally at 60 mg/kg once daily and BKI 1649 at 6 mg/kg once daily for 5 days. BKIs 1294 ($P < .001$), 1369 ($P < .001$), 1534 ($P < .005$), and 1649 ($P < .001$) all showed a significant decrease in the infection level (Figure 3A).

### Comparison of Subcutaneous Versus Oral Administration of BKIs

It is unclear whether the optimal anti-*Cryptosporidium* agent should be absorbed systemically and/or retained in the gastro-intestinal tract [33]. To evaluate subcutaneous administration,

a single 60 mg/kg dose of BKI 1294 or 1534 was administered to uninfected mice. Plasma and fecal levels were measured and compared to oral dose pharmacokinetics (Tables 2 and 3). Subcutaneous delivery of BKI 1294 and 1534 resulted in higher systemic exposure and lower fecal concentrations than oral delivery, even when considering the different doses administered (10 mg/kg orally vs 60 mg/kg subcutaneously in single-dose pharmacokinetics analyses). To compare the efficacy of subcutaneous versus oral dosing, mice were given BKI 1294 or 1534 at 60 mg/kg once daily orally or subcutaneously for 5 days, starting on day 6 after infection (Figure 3B and 3C). Fecal specimens were collected 24 hours after the fifth dose, to measure BKI concentrations (Table 3). The fecal concentration of BKI 1294 was only marginally higher after the fifth dose delivered subcutaneously, compared with the pharmacokinetics of the subcutaneously delivered single dose, while the fecal concentration of BKI 1534 was >10-fold higher after subcutaneous delivery of the fifth dose, compared with pharmacokinetics of the single dose administered subcutaneously (Table 3). BKI 1294 showed a significantly greater reduction in infection when delivered orally, compared with an equivalent dose delivered subcutaneously ($P < .05$; Figure 3B). However, BKI 1534 did not show a significant difference in parasite excretion between groups dosed orally and subcutaneously (Figure 3C).

DISCUSSION

A challenge to the development of effective therapy for *Cryptosporidium* infection is the differential efficiency of parasite clearance in various in vitro and in vivo models [37, 38]. A number of drug candidates have shown good inhibitory activity in vitro but have demonstrated low potency in clinical models and were not further considered [39–41]. In addition to *C. parvum*'s natural resistance to many drug therapies, its characteristic subcellular location on the apical-surface of mammalian intestinal epithelium cells may constitute a barrier to effective drug delivery, preventing drugs from reaching therapeutic concentrations in the parasite [42, 43]. This provides the rationale for targeting enzymes such as *Cp*CDPK1 that phenotypically affect extracellular stages.

Recently, a lack of correlation between anti-*Cryptosporidium* and *Cp*CDPK1 enzyme inhibition for some pyrazolo[2,3-*d*] pyrimidine (PP) compounds was reported, suggesting potential alternative-target activity [34]. The observed c-Src activity for some BKIs supports this potential for off-target kinase activity. BKI 1243 does not have a bumped R1 aryl substituents at the C-3 position. For all other BKIs used in this study, especially with bumped R1, which project into *Cp*CDPK1's enlarged glycine gatekeeper hydrophobic pocket, there is a statistically significant correlation between the *Cp*CDPK1 IC$_{50}$ and the *C. parvum* cellular EC$_{50}$ ($r_{16} = 0.93$; $P < .0001$). This suggests that the R1 groups are important in keeping these PP BKIs on target (Figure 4). Additionally, antisense methods were
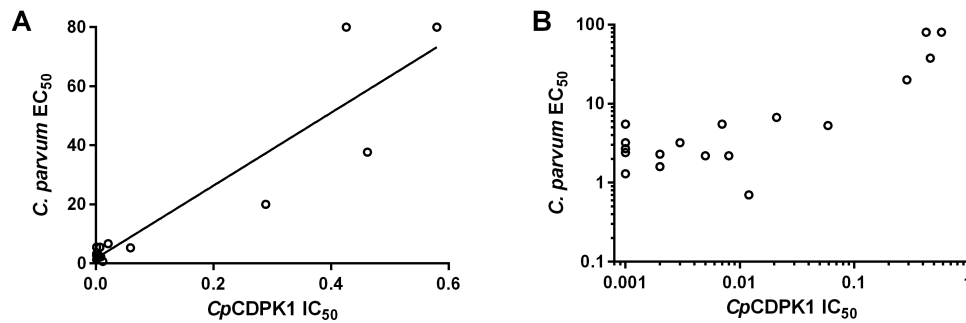
Figure 4. Comparison of the in vitro *Cryptosporidium parvum* calcium-dependent protein kinase 1 (*Cp*CDPK1) 50% inhibitory concentration (IC$_{50}$) to the *C. parvum* 50% effective concentration (EC$_{50}$). *A*, Graphed cellular *C. parvum* EC$_{50}$ versus *Cp*CDPK1 IC$_{50}$ from Table 1. Bumped-kinase inhibitor 1243 was excluded because of its lack of an R1 group. Values show a statistically significant correlation (r$_{16}$ = 0.93; *P* < .0001). *B*, Values graphed on logarithmic scale to better show the separation of IC$_{50}$ values <0.1 mM.

recently used to reduce *Cp*CDPK1 expression [35, 36]. These experiments demonstrated that knocking down expression of CDPK1 significantly decreased parasite invasion and growth, and there was an additive effect with BKIs in reducing the proliferation of *C. parvum* parasites for BKIs 1294 (PP) and 1517 (an aminopyrazole-carboxamide compound) [36]. This potentiation of BKI activity when *Cp*CDPK1 expression is reduced is further evidence that *Cp*CDPK1 is a target of our BKIs. Further chemical-genetic validation experiments are needed to test the hypothesis that *Cp*CDPK1 is the primary target of our selective BKIs.

There is an observed difference between EC$_{50}$ values against *C. parvum* in vitro determined using a reverse transcription–quantitative polymerase chain reaction (RT-qPCR) assay [20, 36] and those determined by the Nluc assay reported here. This could be attributed to the end point readout of the RT-qPCR assay: RNA may degrade faster than the luciferase protein used in the Nluc assay. Thus, delayed degradation of luciferase protein could underestimate compound potency, compared with that determined by RT-qPCR. Nevertheless, luminescence readouts from Nluc-expressing *C. parvum* have been previously demonstrated to correlate with the level of oocyst shedding determined by real-time qPCR using 18S ribosomal RNA primers and DNA extracted from fecal material [24].

The hERG IC$_{50}$ of 10 μM for BKI 1369 was initially a reason for concern in using the compound in humans. Previous studies of hERG suggests that the risk of arrhythmia is reduced for most drugs if the hERG IC$_{50}$ is >30-fold higher than the effective free therapeutic plasma concentration [47]. BKI 1369 displayed a maximum concentration of 0.26 μM at 10 mg/kg, which was 40-fold less than its hERG IC$_{50}$. Additionally, BKI 1369 is 76% protein bound in human plasma (Table 1). The low maximum concentration and unbound fraction of BKI 1369 should mitigate the risks of hERG inhibition [47]. BKI 1294 was efficacious in SCID/beige mice [20] and neonatal mice (Table 1) and had significant therapeutic effects in the calf clinical model of cryptosporidiosis [21]. The efficacy of BKI 1294 was also demonstrated using IFN-γ KO mice infected with Nluc-expressing

C. parvum. BKI 1294 was associated with relapses after treatment in both SCID/beige [20] and IFN-γ KO mouse models. In contrast, no relapses were detected with BKI 1534. The congruence of results across models supports the validity of the IFN-γ KO model for drug development purposes. A likely explanation for recrudescence, especially after high levels of infection are cleared, is the limited immunological resistance of this mouse strain. They will readily reinfect themselves, owing to factors such as oocysts on fur or cage bedding. It is therefore impossible to determine whether infections are recurring because of incomplete clearance or reinfection from grooming, coprophagy, or exposure to fecal matter from cohabitating mice. Future exploration into whether these BKIs will maintain their highly efficacious results in larger mammals is warranted.

Differences in efficacy between the neonatal and immunocompromised adult mice were observed for some compounds, including BKIs 1318 and 1550, which could be due to a number of reasons. These include the transient neonatal versus chronic IFN-γ KO infection and differential physiological characteristics relating to insufficiently developed neonatal organ-specific drug transporters and metabolic systems that may affect drug absorption, distribution, and elimination [44–46]. Furthermore, BKIs tested showed a lack of association between neonatal efficacy and plasma or fecal exposure in adult mice. Nonetheless, intraspecies metabolic barriers may have limited impact on the therapeutic outcome for treatment of cryptosporidiosis with BKIs, based on successful results of recent calf and SCID/beige mouse investigations [20, 21, 35].

In IFN-γ KO mice, there is a lack of correlation between BKI systemic exposure (AUC) and *Cryptosporidium* efficacy (r$_5$ = −0.02; *P* < 1). In contrast, BKIs with high fecal concentrations (Table 2) were efficacious. BKIs 1318, 1550, 1553, and 1649 display the largest plasma AUCs of all compounds tested, yet BKI 1649 proved to be the only compound that was efficacious. BKIs 1318 and 1550 failed to significantly lower infection levels in IFN-γ KO mice, and BKI 1553 was the least effective compound of those tested in neonatal calves [21]. All 3 BKIs showed fecal concentrations in mice below their respective 50%

effective concentrations (Table 1). However, the fecal concentration of BKI 1649 was much higher at 5.76 µM (Table 2). All other BKIs tested that had higher fecal concentrations, including BKIs 1294 (4.4 µM), 1369 (5.2 µM), and 1534 (10.1 µM), were efficacious in mice despite low systemic exposures. This demonstrates that PP-BKI fecal levels correlate with efficacy against *Cryptosporidium* organisms ($r_5 = 0.78$; $P < .05$), in line with the fact that infections localize at the lumen interface within the gut [42, 48]. Since fecal levels are imperfect estimators of exposure in the gastrointestinal tract, future studies are necessary to examine properties beyond plasma and fecal levels to thoroughly explain the pharmacodynamics of successful therapies for cryptosporidiosis.

For efficacy comparison between oral and subcutaneous doses, the BKI 1294 fecal concentration after the fifth dose was similar to its value after a single dose (Table 3). Fecal exposure of BKI 1294 given subcutaneously was around 1 µM, well below the 50% effective concentration for this compound. This offers a possible explanation for the subcutaneous dose being less efficacious than the equivalent oral dose. For BKI 1534, the fecal concentration appeared to rise, with pharmacokinetic analysis of the subcutaneously delivered single dose yielding a concentration of 0.44 µM, compared with 4.76 µM after the fifth dose (Table 3). This potentially explains the lack of significant difference between subcutaneous and oral delivery of BKI 1534 in reducing infection, as subcutaneous administration showed that the BKI concentration in feces increased to therapeutic levels by the end of dosing. This could provide an advantage over BKI 1294 in species for which oral dosing is difficult. Also, the hERG-associated liability is significantly reduced for BKI 1534 (25 µM) as compared to BKI 1294 (0.76 µM). Overall, the 2 compounds are equally efficacious in this mouse model. Given the localization of *Cryptosporidium* infection in the gastrointestinal tract in immunocompetent hosts but biliary and respiratory sites in immunocompromised hosts [49, 50], some systemic absorption is important for optimal efficacy. Further investigations of different dosing routes and formulations are needed, especially in terms of systemic as compared to gastrointestinal exposure.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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