A Cryptosporidium PI(4)K inhibitor is a drug candidate for cryptosporidiosis

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Diarrhoeal disease is responsible for 8.6% of global child mortality. Recent epidemiological studies found the protozoan parasite Cryptosporidium to be a leading cause of paediatric diarrhoea, with particularly grave impact on infants and immunocompromised individuals. There is neither a vaccine nor an effective treatment. Here we establish a drug discovery process built on scalable phenotypic assays and mouse models that take advantage of transgenic parasites. Screening a library of compounds with anti-parasitic activity, we identify pyrazolopyridines as inhibitors of Cryptosporidium parvum and Cryptosporidium hominis. Oral treatment with the pyrazolopyridine KDU731 results in a potent reduction in intestinal infection of immunocompromised mice. Treatment also leads to rapid resolution of diarrhoea and dehydration in neonatal calves, a clinical model of cryptosporidiosis that closely resembles human infection. Our results suggest that the Cryptosporidium lipid kinase PI(4)K (phosphatidylinositol-4-0H kinase) is a target for pyrazolopyridines and that KDU731 warrants further preclinical evaluation as a drug candidate for the treatment of cryptosporidiosis.

Infections that cause diarrhoea are responsible for nearly 800,000 deaths every year, mostly among young children in resource-poor settings. Recently, the apicomplexan parasite Cryptosporidium was found to be one of the leading causes of infectious diarrhoea in children, and infection with this parasite is strongly associated with mortality, growth stunting, and developmental deficits. The major human pathogens causing cryptosporidiosis, C. hominis and C. parvum, infect the epithelial cells of the intestine and, through a mechanism that is not fully understood, trigger severe watery diarrhoeal symptoms. These are particularly long-lasting and often life-threatening in malnourished and immunocompromised children. Nitazoxanide, the only approved drug for the treatment of cryptosporidiosis, has limited efficacy in these most vulnerable patient populations. Cryptosporidiosis is also a well-recognized opportunistic infection in adults with AIDS and transplant recipients. Infection occurs through ingestion of the oocyst stage, which shows remarkable resistance to water chlorination. Therefore, even in countries that apply advanced water treatment, infection is common, and Cryptosporidium is the cause of 50% of disease outbreaks linked to recreational water use in the USA. The search for cryptosporidiosis therapeutics has been hindered by the many technical challenges faced when working with this notoriously intractable parasite. Here we establish a drug discovery screening process for cryptosporidiosis that combines phenotypic in vitro assays with novel animal models that take advantage of transgenic parasites.

**Cryptosporidium compound screen**

To discover new treatments for cryptosporidiosis, we assembled a set of 6,220 compounds with known activity against various protozoan parasites and screened them against C. parvum in a high-content imaging infection assay in HCT-8 cells (see Supplementary Information). Notably, many anti-malarial agents (spiroindolones, cyclomarins, and imidazolopiperazines) lacked activity against Cryptosporidium; however, 154 compounds showed >60% growth inhibition at 5 μM. Secondary screening using a novel cytopathic effect (CPE)-based C. parvum assay confirmed several scaffolds, with imidazopyrazines and pyrazolopyridines showing sub-micromolar cellular activity (Fig. 1a–d and Table 1; structures provided in Extended Data Fig. 1). We evaluated about 200 pyrazolopyridine analogues and found correlation between activity against C. parvum and Plasmodium falciparum (r² = 0.702; Fig. 1d), suggesting that the mechanism of action of pyrazolopyridines is conserved between these two parasites. No such correlation was observed with toxicity against HepG2 (r² = 0.071) (Extended Data Fig. 2). C. hominis is responsible for most clinical infections. We thus evaluated a subset of pyrazolopyridine analogues against C. hominis, and found potency comparable to that against C. parvum (r² = 0.872; Fig. 1e and Table 1).

**Pyrazolopyridines inhibit CpPI(4)K** Knowing that pyrazolopyridines and imidazolopyridines exert their anti-malarial activity through inhibition of the Plasmodium lipid kinase PI(4)K, we searched for potential Cryptosporidium orthologues. The genomes of both C. parvum and C. hominis encode multiple putative lipid kinases, and the PI(4)K catalytic domain of cgdb_4500 and its C. hominis homologue Chro.80518 show 71.8% amino-acid sequence similarity to P. falciparum PI(4)K. We expressed cgdb_4500 in insect cells and purified the protein (CpPI(4)K), which displays phosphatidylinositol kinase activity with a Michaelis constant (Km) for ATP and phosphatidylinositol of 3 and 0.4 μM, respectively (Extended Data Fig. 3). Using this assay, we showed that the imidazopyrazine KDU691 and the pyrazolopyridine KDU731 are potent inhibitors of CpPI(4)K.

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KDU731 warrants further preclinical evaluation as a drug candidate for cryptosporidiosis. In neonatal calves, a clinical model of cryptosporidiosis that closely resembles human infection. Our results suggest that the Cryptosporidium lipid kinase PI(4)K (phosphatidylinositol-4-0H kinase) is a target for pyrazolopyridines and that KDU731 warrants further preclinical evaluation as a drug candidate for the treatment of cryptosporidiosis.

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enzymatic activity with half-maximal inhibitory concentration (IC50) values of 17 and 25 nM, respectively (Fig. 1f). Both compounds have a >50-fold selectivity window against the human PI(4)K III homologue (Extended Data Table 1).

When we measured diverse imidazopyrazine and pyrazolopyridine analogues against CpiPi(4)K enzymatic activity and C. parvum growth in cells, we found a tight correlation ($r^2 = 0.902$) (Fig. 1g), suggesting that the anti-Cryptosporidium activity is directly mediated by CpiPi(4)K inhibition. Further genetic and structural insights are needed to unambiguously establish P14(K) as the target. However, we note that the Plasmodium Pl(4)K inhibitors diaryl-aminopyridine (MMV390048) and quinoxaline (BQR695), which are inactive against C. parvum and C. hominis, do not inhibit CpiPi(4)K enzymatic activity (Fig. 1f).

Finally, mechanistic studies revealed an increased Km for ATP in the presence of KDU731 (Fig. 1h), suggesting ATP-competitive inhibition similar to that previously observed for the Plasmodium enzyme.12 Additional structure–activity relationship analysis of pyrazolopyridines against Cryptosporidium is found in the Supplementary Information. Taken together, our data suggest that pyrazolopyridines are inhibitors of CpiPi(4)K that bind to the ATP-binding site of the enzyme with a favourable selectivity window over human PI(4)K.

### Pharmacokinetic properties of KDU731

The most urgent need for effective cryptosporidiosis treatment is among children under the age of 2 years. A very safe drug profile is thus a key component of the target product profile.18,19 KDU731 has a selectivity index of more than 100 (half-maximal cytotoxicity concentration (CC50) of HepG2 $= 15.61 \mu M$ and half-maximal effective concentration (EC50) of C. parvum CPE $= 0.11 \mu M$) (Extended Data Table 1). In a battery of safety pharmacology assays, the compound does not show intrinsic risks for cardiotoxicity, mutagenicity, clastogenicity, or phototoxicity, and it does not bind significantly to a panel of human anti-targets (Extended Data Tables 1 and 2).

KDU731 safety was further evaluated in a 2-week toxicology study in rats using a solid dispersion formulation to maximize systemic exposure (~25-fold). In this study, no significant histopathological changes and only minor changes in clinical chemistry and haematology were observed (slight elevation of cholesterol and phosphate levels).

### Table 1 | Activity of pyrazolopyridine analogues and other known PI(4) kinase inhibitors

| Compound   | CpiPi EC50 (M) | CpiPi IC50 (M) | CpiPi IC50 (M) | CpiPi IC50 (M) | CpiPi IC50 (M) | CpiPi IC50 (M) | CpiPi IC50 (M) | CpiPi IC50 (M) |
|------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| KDU731     | 0.107 ± 0.039  | 0.063 ± 0.028  | 0.130 ± 0.074  | 0.025 ± 0.004  | 0.003 ± 0.001  | 15.62 ± 8.621  |
| KDU691     | 0.096 ± 0.044  | 0.054 ± 0.029  | 0.082 ± 0.017  | 0.017 ± 0.012  | 0.108 ± 0.061  | 27.29 ± 14.169 |
| MMV390048  | 12.792 ± 6.264 | 13.422         | 11.85 ± 2.758  | 8.335 ± 2.355  | 0.042 ± 0.022  | 26.156 ± 16.063|
| BQR695     | 11.837 ± 1.889 | 8.344          | 8.565          | 7.305 ± 0.841  | 0.118 ± 0.055  | 26.711 ± 13.870|
| Nitazoxanide | 14.286 ± 7.127 | 2.927 ± 0.808  | >20.000        | >10.000        | >10.000        | 12.749 ± 2.378 |
| KDU370     | 1.540          | ND             | 2.543          | 0.212          | 0.074 ± 0.032  | >50.000        |
| KDZ464     | 0.113 ± 0.072  | 0.232          | 0.119          | 0.003          | 0.005 ± 0.001  | 21.222 ± 10.594|
| LNN134     | 1.951 ± 0.340  | 1.031          | 1.937          | 0.488          | 0.126 ± 0.115  | 40.025 ± 17.278|
| LMW740     | >20.000        | >20.000        | >20.000        | >10.000        | 0.521          | >50.000        |
| KEL204     | >20.000        | >20.000        | >20.000        | 3.701          | 1.432          | >50.000        |

CpiPi, C. parvum; CH, C. hominis; PI, P. falciparum; HCl, high-content imaging assay; ND, not determined. Data shown are means ± s.e.m., n = 3 biological replicates.
Figure 2 | KDU731 has potent activity against Cryptosporidium in immunocompromised IFN-γ knockout mice. a, Mice were infected with 10,000 C. parvum UGA1 Nluc or UGA2 Fluc oocysts. Parasite load in the faeces was determined by measuring faecal Nluc activity and parasite tissue load was quantified by whole-animal imaging of Fluc activity. Different 7-day treatment courses are indicated in red. Faecal luciferase measurements of individual mice with treatment initiated after 3 (b) or 11 (c) days of infection (red; vehicle control shown in black), n = 5 and n = 9 mice respectively, representative of two biological replicates for b and c. d, Histology of the ileum of infected mice (shown in c) after 1 week of KDU731 treatment compared with vehicle-treated control on day 18 (n = 3 biological replicates, representative images shown here). Note numerous intracellular parasite stages (white arrowheads) and extracellular oocysts (black arrowhead) in the control, absent in the treated mice. Vehicle-treated animals showed disorganized columnar epithelial cells and loss of brush border compared with KDU731-treated mice. White box indicates section shown at higher magnification to the right. Mice (n = 5) infected with UGA2 Fluc were treated on day 7 with 10 mg per kg (body weight) of KDU731 (red, e and g), 100 mg per kg (body weight) nitazoxanide (red, f, also see Extended Data Fig. 6), or vehicle (black, e and f) for 1 week. Animals were monitored by whole-animal imaging and a radiance scale and quantification of total flux in photons per second is shown. *Animals shown on the baseline were below the level of detection.

KDU731 treatment of infected mice

Current assessment of cryptosporidiosis treatments relies on laborious methods to quantify the parasite in animals. We used genetically modified parasites to build more facile models. We established the EC50 of KDU731 for transgenic parasites8 in HCT-8 using Nanoluciferase (Nluc) as a readout and found it comparable to the EC50 for wild-type parasites (Extended Data Fig. 4a). Next, we inoculated 6- to 8-week-old C57BL/6 interferon-γ (IFN-γ)-knockout mice with 10,000 oocysts and monitored infection by following parasite-derived luciferase activity in the faeces (Fig. 2a). Infected mice were treated orally with KDU731 in suspension formulation and parasite load was measured in the pooled faeces by Nluc and quantitative PCR (qPCR) assay with a high degree of correlation (Spearman coefficient = 0.786; two-tailed P = 0.048). The Nluc assay has a small sample requirement (20 mg) and moderate-to-low oral bioavailability (37% in rodents, 9% in non-human primates). As Cryptosporidium primarily infects the intestinal epithelium, we reasoned that systemic exposure may not be required for efficacy and that limiting systemic exposure may further enhance the safety margin of a cryptosporidiosis drug.
was initiated when calves showed severe diarrhoea (faecal consistency treatment with KDU731 (5 mg per kg (body weight) every 12 h for 7 days) of severe diarrhoea (significantly fewer oocysts in their stool (untreated calves.

Within 48 h of birth, calves were challenged 13 neonatal calves with 5 × 10^6 C. parvum infection, resulting in fulminant diarrhoea and faecal oocyst shedding, between day 2 and day 4 after infection. All calves tolerated KDU731 treatment without compound-related abnormalities, and treated calves shed significantly fewer oocysts than vehicle-treated calves within 3 days of treatment (P < 0.0001 on day 3) (Fig. 3b). Treated calves suffered fewer days of severe diarrhoea (two-tailed P = 0.006) and were significantly less dehydrated (two-tailed P < 0.0001) than controls (Fig. 3c, d). Resolution of clinical signs started as early as 24 h after treatment was initiated. Within 48 h of treatment, six out of seven calves showed no signs of dehydration, and within 72 h of treatment five of six calves had resolution of severe diarrhoea (Extended Data Fig. 7). KDU731 displayed limited systemic exposure in calves with a maximum serum concentration (C_max) and an absorption (area under the concentration–time curve, AUC) of 0.228 ± 0.08 μM and 1.9 ± 1.8 μM h⁻¹ respectively (Fig. 3e and Extended Data Table 3), confirming that substantial systemic exposure may not be required for parasite clearance and resolution of clinical illness.

**Conclusions**

Our studies define the pyrazolopyridine KDU731 as a promising anti-cryptosporidial drug candidate that is active against both C. parvum and C. hominis. Unlike nitazoxanide, KDU731 demonstrated in vivo efficacy in immunocompromised mice. Additionally, treatment in neonatal calves, which closely matches the pathophysiological and pharmacological challenges faced in the treatment of young malnourished children, led to a significant decrease in parasite shedding and rapid resolution of diarrhoea and dehydration. Our lead candidate, KDU731, displays good anti-cryptosporidial activity and meets a broad range of safety and pharmacology criteria required for a much-needed novel cryptosporidiosis therapeutic intervention. Further safety and pharmacological preclinical evaluation is currently ongoing to support the initiation of human clinical trials.
Online Content

Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information

is available in the online version of the paper.

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Author Contributions

U.H.M., S.V., J.A.Z., B.S., and T.T.D. conceived and designed the study; B.S. wrote grant applications with contributions from U.H.M.; U.H.M., A.T.C., and G.M.C.B. developed C. parvum screening assays; C.G.N. and S.H.L. developed enzyme assays; C.B. analysed P. falciparum EC₅₀ data; U.H.M., P.G., and T.T.D. assembled the screening library; R.R.K. and B.Z. performed compound synthesis; U.H.M., B.Z., and J.W. analysed the structure–activity relationship; S.B.L. and F.B. analysed in vivo pharmacokinetics data; L.Z. optimized formulation; U.H.M., G.F., F.J.L., and T.T.D. analysed in vivo efficacy and toxicology results; S.V., A.S., and B.S. designed mouse models based on transgenic parasites; S.V., A.S., and B.S. designed mouse models based on transgenic parasites; S.V., A.S., and B.S. designed mouse models based on transgenic parasites; S.V., A.S., C.F.B. and G.T.H. validated mouse models; G.T.H., S.V., and C.F.B. tested compounds; J.A.Z. developed the calf model and analysed calf data; T.L.S. executed the calf model; S.N. conducted anatomic pathology reviews for efficacy and toxicity; L.G.B. developed and executed calf stool analytics; and B.S., S.V., U.H.M., and T.T.D. wrote the manuscript with contributions from J.A.Z., A.T.C., C.G.N., and S.B.L.

Author Information

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Reviewer Information

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PI(4)K enzymatic assay. The CpPI(4)K enzyme assay was described earlier22. Briefly, 1-α-phosphatidylinositol (Avanti Polar Lipids), dissolved in 3% octylglucoside (Roche Diagnostics), was used as lipid substrate using a Transcreener ADP, FP detection kit (BellBrook Labs) in black, solid 384-well plates (Corning 3575). The final assay volume was 10 μl and contained 3 nM CpPI(4)K in 10 mM Tris, pH 7.5, 1 mM DTT, 3 μM ATP, 5 mM MnCl2, 0.05% Triton X-100, and 100 μM phosphatidylinositol/octylglucoside. The enzyme reaction was performed for 50 min at room temperature and stopped by adding 10 μl of detection mix containing 50 nM HEPEI, pH 7.5, 400 μM NaCl, 20 μM EDTA, and 0.002% Brij-35, 2 mM AMP, Alexa Fluor 633 tracer, and 20 μg ml−1 ADP antibody (BellBrook Labs). Fluorescence polarization measurements were performed on an Infinite M1000 plate reader (Tecan) with excitation and emission wavelengths of λex = 635 nm and λem = 680 nm (20 nm bandwidth). IC50 values were calculated using GraphPad Prism software.

Solubility, permeability, in vitro metabolic stability, and in vitro safety assessment. Solubility was measured using a miniaturized shake-flask approach and stream-lined high-performance liquid chromatography (HPLC) analysis as described earlier23. Parallel artificial membrane assays were performed using a standard protocol23. The metabolic stability in liver microsomes was determined using the compound depletion approach and quantified by liquid chromatography/mass spectrometry (LC/MS). The assay measures the rate and extent of metabolism as determined by the disappearance of the parent compound, which allows the determination of in vitro half-life (t1/2), intrinsic clearance, and the prediction of metabolic clearance in various species20. Cardiotoxicity and mini-ami genotoxicity risk was measured as previously described24. All assays for binding to proteins known to bear potential safety liabilities in humans were high-throughput competitive binding assays using specific radiolabelled ligands25. The phototoxicity assay was performed following OECD Guidelines for Testing of Chemicals 432 (in vitro 33 NU photon toxicity Assay).

Cytochrome P450 analysis. KD7U31 was subjected to CYP450 inhibition analysis using three different isozymes20. The compounds were assessed for time-dependent inhibition using CYP3A4 (ref. 30). The CYP induction assay was performed using a PXR receptor; binding was assayed using a LanthaScreen TR-FRET PXR (SXR) competitive binding assay kit from Invitrogen26. In vivo pharmacokinetic analysis. Rodent in vivo pharmacokinetic analysis was conducted using non-randomized CD-1 female mice (n = 4, 6–8 weeks old) and Wistar rats (n = 4, 6–8 weeks old). Pharmacokinetic studies in monkeys (n = 3) were performed in rhesus macaques as described27. Neonatal calf pharmacokinetic studies were performed as part of the efficacy study on day 1 and day 7 of treatment. All procedures involving animals were reviewed and approved by the respective institutional animal care and use committees. No statistical methods were used to predetermine sample size. Sample size was determined on the basis of the minimum number of animals required for good data distribution and statistics. Blinding was not possible in these experiments but animals were selected randomly for each group. KD7U31 was formulated in suspension formulation for per os dosing and solution formulation for intravenous dosing as described in Extended Data Table 3. For rat toxicology studies, KD7U31 was formulated in solid dispersion. KD7U31 (20%) and the required excipients (37.5% Soluplus, 37.5% Eudragit E PO, and 5% SLS) were dissolved in ethanol/dichloromethane (v/v = 1:1) at a total solid concentration of 10 g·l−1 by sonication and heating to 50°C. The solution was spray dried using a Buchi B290 Mini spray dryer with an inlet temperature of 75°C, aspirator set to 100%, and pump to 35%, followed by overnight drying under vacuum at 40°C. The amorphous nature was confirmed by X-ray powder diffraction analysis and powder was stored at 4°C. Solid dispersion powder suspension was prepared freshly in 0.5% HPMC in 50 mM acetate buffer (pH 4.7) at 10 mg · ml−1 of KD7U31 equivalent to solid dispersion powder 50 mg · ml−1 and rats were dosed within 1 h of preparation. Blood samples for pharmacokinetic studies were collected between 0 and 24 h post-dose. Compound plasma concentrations were determined by LC/MS. Plasma samples from pharmacokinetic studies were extracted with acetonitrile:methanol:acetic acid (90:9.8:0.2) containing warfarin as an internal standard, using an 8.8:1 extractant to plasma ratio. Analyte quantitation was performed by LC/MS/MS. Liquid chromatography was performed using an Agilent 1200 HPLC system, with an Agilent Zorbax Phenyl (3.5 μm, 4.6 mm × 75 mm) column at an oven temperature of 45°C, coupled with aAPI4000 triple quadrupole mass spectrometer (Applied Biosystems). Pharmacokinetic parameters were determined using Watson LIMS software, by non-parametrical analysis.

Rat toxicity study. KD7U31 solid dispersion powder suspension was prepared freshly in 0.5% HPMC in 50 mM acetate buffer (pH 4.7) at 10 mg · ml−1 and was orally administered to five male Wistar rats at a daily oral dose of 30 or 100 mg per kg (body weight) per day for 2 weeks. Five control animals were treated with vehicle only. Rats were obtained from Shanghai SLAC Animal and subjected to...
3 days of quarantine and acclimatization before study begin. All animals were subjected to daily clinical observation, and body weight and food consumption were determined for all animals enrolled in the study. Clinical laboratory evaluations (haematology and clinical chemistry) were performed at the scheduled necropsy on day 15. Organs were examined for gross pathology and weighed before fixation and preparation for histology. Samples from organs and tissues prepared from animals assigned to control and high-dose groups were examined microscopically. Specifically, the heart, pancreas, gastrointestinal tract (oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum), kidney, liver, spleen, lung, testes, epididymis, adrenals, and thymus were examined for histopathological changes.

Engineering of a *C. parvum* transgenic parasite strain expressing red-shifted Fluc. The 5′ untranslated region (UTR) and 3′ UTR of the *C. parvum* actin gene were amplified from parasite genomic DNA and ligated into the KpnI/Clal and SpeI/BamHI sites of plasmid TSK-En-Nluc-Neo-TK respectively. The coding sequence for red-shifted luciferase was amplified from pTubE9 vector (a gift from M. Meissner, University of Glasgow, UK) and cloned into SalI/Nhel restriction sites replacing Nluc. A 0.408-base pair fragment of the 5′ TK flanking, the Nluc, and a ribosomal 3′ UTR were inserted upstream of the 5′ actin UTR using Gibson Assembly cloning (New England Biolabs). The final vector, along with the Cas9 plasmid containing a TK guide RNA (GAAGAATACATTCTTACAAGG) targeting the 3′ end of the tk gene, was used to transfect *C. parvum* sporozoites. Sporozoites were delivered by surgical into the small intestine of mice. The transgenic parasite strain expressing red-shifted Fluc (*C. parvum* IOWA II oocysts purchased from Sterling Parasitology Laboratory, University of Arizona, USA) were injected subcutaneously with 125 mg per kg (body weight) d-luciferin (Gold Laboratories, Hercules, California, USA). Five minutes after luciferin injection, mice were anaesthetized in an induction chamber using 3% isofluorane through individual nose cones. Images were acquired using a setting of F-stop = 1/16, binning = medium, and an exposure time of 5 min. Regions of interest were selected for each mouse and total flux (photons per second) was quantified using IVIS Lumina LII Imaging 4.0 Software (Caliper Life Sciences). To validate measurements of parasite tissue burden, mice infected for 1 week were imaged, killed, and the small intestine was removed and cut into 12 (1-cm) segments, and flushed with PBS. Sections were imaged three times in PBS with n-luciferin, after which genomic DNA was isolated from each segment using a QIAamp DNA minikit (Qiagen, Valencia, CA). qPCR was performed using the parameters described above and parasite burden was established against a standard curve of samples with known parasite DNA content.

Histology of intestinal tissue. Mice treated with KDU731 or control formulation were killed and intestinal tissue was collected within 15 min of death. Sections of the small intestine were taken from the 1–2 cm region anterior to the caecum, flushed with PBS, and fixed overnight in 10% buffered formalin. Fixed samples were embedded in paraffin and 4 μm sections were cut (RM225 Microtome, Leica, Buffalo Grove, Illinois, USA). Sections were de-paraffinized and stained with haematoxylin and eosin.

Neonatal calf efficacy study. All calves used in this study were cared for in compliance with the Washington State University Institutional Animal Care and Use Committee. Sample size was calculated assuming that 85% of treated calves had resolution of clinical illness by the end of the treatment period (48 h after administration of treatment number 14) compared with 15% of control calves. Assuming a type I error risk of 5% and a type II error risk of 80%, seven calves were needed in the treatment group, plus two positive controls. In the event of calf death or removal from the study, an additional four calves were added to the control group (n = 6). Sample size was calculated using Epi Info. Fifteen Friesian–Holstein bull calves were enrolled in November 2015 and February 2016. At birth, all calves enrolled in November were randomized to treatment with KDU731 (n = 7), positive infection control (n = 2), and negative infection control (n = 1). The five calves enrolled in February were positive controls. The perineum of the dam was cleaned with povidone–iodine scrub, and calves were delivered onto single-use plastic sheets to prevent exposure to environmental pathogens. Calves with abnormal physical examination findings and those weighing less than 29.5 kg at birth were excluded. Enrolled calves received 41 ± 50 g kg⁻¹ commercial colostrum replacer (Land O’Lakes) and a 3 mL subcutaneous injection of vitamin E and selenium (Merck Animal Health). Calves were then transported from the commercial dairy farm to Washington State University where they were housed in individual box stalls in a BSL-2 facility. Shatter-proof mirrors were provided for enrichment. Within 48 h of birth, blood samples were collected and evaluated for adequate passive transfer of colostral immunity. Calves were offered a commercial 20% protein/20% fat non-medicated milk replacer (Land O’Lakes) every 12 h via nipple bucket. At each feeding, calves were fed an average of 8.8 g of dry matter per kilogram of birth weight for the duration of the study. Water was provided ad libitum. All calves randomized to treatment or positive control groups were experimentally challenged within the first 48 h of life with 5 × 10⁶ *C. parvum* oocysts (Iowa II, Bunch Grass Farm, ID) through the rigid portion of an orogastric feeding tube. Oocysts were within 1 month of isolation and were cleaned of the small intestine were taken from the 1–2 cm region anterior to the caecum, flushed with PBS, and fixed overnight in 10% buffered formalin. Fixed samples were embedded in paraffin and 4 μm sections were cut (RM225 Microtome, Leica, Buffalo Grove, Illinois, USA). Sections were de-paraffinized and stained with haematoxylin and eosin.

Mouse model following birth, a faecal sample was collected directly from the rectum every 24 h. A complete physical examination was performed every 12 h, and clinical data including temperature, pulse, and respiratory rate were recorded. Clinical data were evaluated on a scale of 1 (normal) to 3 (severe) in accordance with previous described methods (see rubric in Supplementary Information)²³,²⁴. KDU731 was prepared as a 5 mg ml⁻¹ suspension formulation in 0.5% (w/v) methylcellulose and 0.5% w/v Tween-80 in water, and KDU731 treatment was initiated when a calf began shedding oocysts and had a faecal consistency score ≥ 3. Calves were induced to suckle and then KDU731 was orally given via an oral dosing syringe. Calves were treated every 12 h for 7 days at a dose of 5 mg kg⁻¹ birth weight at least 2 h after feeding. Pharmacokinetic sampling was conducted on day 1 and day 7 of treatment. Blood was drawn before and at multiple time-points after administration of the treatment. A faecal sample was collected 1 h and 12 h after administration. On days 2–6 of treatment, blood was collected before KDU731 administration. On day 3 of life, a faecal sample was tested for *E. coli* K99, and on day 7 for *Salmonella*, rotavirus, and coronavirus. Of the seven treatment calves, two (N101 and N107) were randomly selected to be euthanized 24 h after administration of the final KDU731 treatment. A positive control calf (N104) was also euthanized at the same day 15.
time. Euthanasia was by captive bolt and induction of bilateral pneumothorax to avoid confounding histological findings. Calves were submitted for necropsy to the Washington State Animal Disease Diagnostic Laboratory. Samples of the liver, spleen, kidney, and small intestine were collected for histological analysis. The remaining calves continued in the study until the cessation of faecal oocyst shedding as determined by immunofluorescence microscopy (Merifluor, Meridian Diagnostics). Upon cessation of faecal oocyst shedding (two consecutive negative faecal samples over 48 h), calves were euthanized and submitted to the Washington State Animal Disease Diagnostic Laboratory for necropsy.

Oocysts counts were interpolated by qPCR at the Cornell Animal Health Diagnostic Center using serial dilutions of commercially purified *C. parvum* oocysts (Waterborne, New Orleans, Louisiana, USA). Total nucleic acid was extracted from supernatants of 200 mg of faecal sample, oocyst suspension, or negative control homogenized in 400 μl of PBS using a magnetic-bead-based automated procedure (AM1840, Applied Biosystems, Foster City, California, USA). An exogenous control (MS2 phage) was added to the lysis buffer to control for PCR inhibition34. qPCR for *Cryptosporidium* spp. 18S rRNA was performed on an Applied Biosystems 7500-Fast platform using commercial master mix (ToughMix, Quantabio) and oligonucleotides previously described35. This count was standardized by the faecal dry weight percentage. A 5–10 g portion of each original faecal sample was dried at 108 °C for a minimum of 24 h (Squaroid Vacuum Oven, Labline, India) and weighed34. Data were analysed using descriptive and inferential methods. A Shapiro–Wilk test was used to determine whether data were non-Gaussian. Depending on the distribution of data, continuous variables were evaluated using a Student's t-test, analysis of variance, or a Wilcoxon rank-sum test. Analysis of variance was used to assess differences in faecal oocyst counts and faecal consistency score between the KDU731-treated and untreated calves. The effect of comorbidity on outcomes was evaluated using a Student's t-test. Data were analysed using JMP Pro 11.0 (SAS Institute).

**Data availability.** Source data analysed for mouse and calf infection experiments shown in Figs 2 and 3 are included as Microsoft Excel files in the Supplementary Information. Requests for compounds (Novartis Institute for Tropical Diseases) and transgenic parasite strains (University of Georgia, Athens) are subject to a Material Transfer Agreement. All other data are available from the corresponding authors upon reasonable request.

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Extended Data Figure 1 | Structures of the pyrazolopyridines and other known PI(4) kinase inhibitors. Compounds described in Table 1. Important structural determinants required for anti-Cryptosporidium activity in pyrazolopyridines are shown in blue.
Extended Data Figure 2 | Anti-Cryptosporidium activity does not correlate with mammalian cell toxicity. Correlation of C. parvum cytopathic effect versus HepG2 cytotoxicity assay for selected pyrazolopyridine and imidazopyrazine analogues along with BQR695 and MMV390048. Data shown here are geometric mean EC₅₀ values, with at least two biological replicates.
Extended Data Figure 3 | Recombinant *C. parvum* cgdk8_4500 shows phosphatidylinositide kinase activity. *C. parvum* cgdk8_4500 was expressed in insect cells using a Baculovirus system and recombinant enzyme was purified. A Michaelis–Menten plot of phosphatidylinositide kinase reaction with 3 nM CpPI(4)K enzyme at varying ATP concentrations is shown. Data shown here are a representative graph of two independent biological replicates.
Extended Data Figure 4 | KDU731 inhibits *C. parvum* Nluc parasites *in vitro and in vivo*. **a**, EC₅₀ determination of KDU731 against UGA1 Nluc transgenic parasites grown in HCT-8 cultures using luciferase activity as read out. Representative data are shown, three technical replicates. **b**, Mice (*n* = 5) were infected with 10,000 UGA1 Nluc oocysts and treated orally 3 days after infection with 1, 5, or 10 mg per kg (body weight) KDU731 for 1 week. Faecal oocyst load was determined by measuring parasite luciferase activity (b) or parasite DNA by qPCR (c) in faeces pooled from entire cage of five mice (20 mg faeces for Nluc and 100 mg for PCR assay). **b**, **c**, Means for three technical replicates are shown. Error bars, s.d. Pooled Nluc experiments for vehicle and 10 mg per kg (body weight) dose were repeated in three biological replicates and a representative result is shown.
Extended Data Figure 5 | Parasite intestinal load measured by qPCR correlates with faecal shedding and tissue luminescence. a, Mice (n = 4) were infected with 50,000 UGA2 FLuc oocysts and imaged after 1 week. Mice were killed and the small intestine was resected and imaged (representative image shown). Infection of the intestine ranged in intensity from heavy in the ileum to more moderate in the jejunum and caecum (see radiance scale bar for comparison). Intestines were cut into 12 segments and the luminescence of each segment was recorded. b, qPCR analysis of intestinal segments was performed in triplicate and plotted against the respective luminescence measurements. Regression analysis found robust correlation of tissue luminescence and PCR for parasite DNA, with $r^2 = 0.8$. c, Mice were infected with 10,000 UGA2 FLuc oocysts, and 7 days after infection animals were treated daily for a week with vehicle or 10 mg per kg (body weight) KDU731. Whole-animal imaging during the treatment period is shown in Fig. 2g. Faecal oocyst load was determined by measuring parasite DNA by qPCR in faeces pooled from a cage of five mice. Error bars, s.d.
Extended Data Figure 6 | Nitazoxanide does not reduce intestinal parasite load in IFN-γ knockout mice. Mice (n = 5) were infected with 10,000 UGA2 FLuc oocysts, and 7 days after infection animals were treated daily for a week with 100 mg per kg (body weight) nitazoxanide or vehicle. Mice were monitored by whole-animal imaging. Radiance scale shows total flux in photons per second.
Extended Data Figure 7 | Effect of KDU731 on severity of diarrhoea and dehydration in the neonatal calf model of cryptosporidiosis. Severity of diarrhoea and dehydration in individual calves challenged with $5 \times 10^7$ C. parvum oocysts. Infected calves were treated with vehicle ($n = 6$) or with KDU731 ($n = 7$); $n$ represents the number of calves. Every 12 h, calves were stimulated to defecate, faecal consistency was evaluated, and hydration status was assessed. Faecal consistency and hydration scores were assigned according to the study rubric (see Supplementary Information). The schematic representation shows the faecal consistency (a) and hydration scores (b) throughout the drug treatment period. Faecal consistency and hydration began to improve within 48 h of initiating treatment with KDU731.
## Extended Data Table 1 | Physicochemical properties and safety profiling data for KDU731

| Properties                     | KDU731 |
|-------------------------------|--------|
| Molecular Weight (Da)        | 396.41 |
| Solubility (pH 6.8) (μM)     | 20     |
| Lipophilicity (logP)         | 1.7    |
| PAMPA (% calc fraction absorbed) | 34.7   |
| Caco2 permeability (ratio B-A/A-B) | 2.42   |
| in vitro clearance (M/R/D/Mk/H) | - / 6 / < 0.8 / <4 / 12 |
| Microsomal CLint [μl/min×mg]  | 50 / 29 / 36 / 39 / 13 |
| Hepatocytes [μl/min/million cells] | - / 6 / < 0.8 / <4 / 12 |

### Cellular activity (μM)

- Cytotoxicity HepG2 (CC<sub>50</sub>) 15.6
- C. parvum / C. hominis (EC<sub>50</sub>) 0.10 / 0.13
- HepG2 CC<sub>50</sub> / C. parvum EC<sub>50</sub> ratio (SI) > 100

### % Plasma protein binding

|M/R/D/H| - / 87.7 / 70.8 / 79.1|

### Human lipid and related kinase enzyme IC<sub>50</sub> (μM)

- PI(4)β 1.4
- PI(3)δ 1
- PI(3)β 1.9
- PIK(3)Cy 0.88
- PIK(3)Cφ 0.39
- VPS34 >9.1
- mTOR 4.3

### CYP P450 isoforms inhibition (IC<sub>50</sub> μM)

- Reversible 3A4 >50
- Reversible 2D6 >50
- Reversible 2C9 >4.8
- Time dependent inhibition 3A4 Negative
- CYP induction, PXR functional assay > 10

### Cardiotoxicity

- hERG Binding (μM) >30
- Q-Patch IC<sub>50</sub> (μM) 28
- Patch Clamp Nav1.5 Quattro IC<sub>50</sub> (μM) >50

### Genotoxicity

- Mini-AMES Negative
- Micronucleus test (MNT) Negative

### Phototoxicity (PIF values)

- Negative (3.1)

### Safety pharmacology profiling

- (selected receptors, ion-channels, transporters, kinases etc) No significant binding/inhibition

*Details in Extended Data Table 2. Data presented here is from assays repeated at least 2 times.
Extended Data Table 2 | Effect of KDU731 on radio-ligand binding to a panel of human recombinant receptors and pharmacologically relevant proteases/kinases

| Binding assay* | IC₅₀ (µM) | Kinase assay | IC₅₀ (µM) |
|----------------|-----------|--------------|-----------|
| Adenosine 1 receptor | >30 | cABL, T315 | >10 |
| Adenosine 2A receptor | >30 | ALK, T315 | >10 |
| Adenosine 3 receptor | >30 | Aurora-A | >10 |
| Adenosine transporter | >30 | AXL | >10 |
| Adrenergic 1 receptor | >30 | BTK | >10 |
| Adrenergic ß2 receptor | >30 | CDK2A | >10 |
| Adrenergic ß2C receptor | >30 | CDK4D1 | >10 |
| Angiotensin II AT1 receptor | >30 | EGFR | >10 |
| Benzodiazepine receptor | >30 | EPHA4 | >10 |
| Cholecystokinin A receptor | >30 | EPHB4 | >10 |
| Cholecystokinin B receptor | >30 | FGFR3 | >10 |
| COX-1 assay* | >30 | GSK3B | >10 |
| COX-2 assay | >30 | IGF1R | >10 |
| Dopamine D2 receptor | >30 | INSR | >10 |
| Dopamine D3 receptor | >30 | JAK1 | >10 |
| Dopamine transporter | >30 | JAK2 | 6.7 |
| Endothelin A receptor | >30 | JAK3 | >10 |
| Ghrerin receptor | >30 | KOR | >10 |
| Histamine H1 receptor | >30 | KIT | >10 |
| Histamine H3 receptor | >30 | LCK | >10 |
| Melanocortin MC3 receptor | >30 | MAP3K8 | >10 |
| Monoamine oxidase A | >30 | MAPK14 | >10 |
| Motilin receptor | >30 | MAPK1 | >10 |
| Muscarinic M1 receptor | >30 | MET | >10 |
| Muscarinic M3 receptor | >30 | PDGFRα | >10 |
| Nicotinic (CNS) receptor | >30 | PDK1 | >10 |
| NMDA channel site receptor | >30 | PKN1 | >10 |
| Norepinephrine transporter | >30 | PKN2 | >10 |
| Opiate δ receptor | >30 | PRKCA | >10 |
| Opiate κ receptor | >30 | PRKCA | >10 |
| Phosphodiesterase 3 | >4.5 | RET | >10 |
| Phosphodiesterase 4D | >30 | ROCK2 | >10 |
| Serotonin 5-HT2C receptor | >30 | SYK | >10 |
| Serotonin 5-HT3 receptor | >30 | TYK2 | >10 |
| Serotonin transporter | >30 | ZAP70 | >10 |
| Vasopressin V1a receptor | >30 | | |
| **Protease assays** | | | |
| Caspases 3 | >30 | | |
| Cathepsin D | >30 | | |
| Matrix Metalloproteinases | >30 | | |
| MMP08 | | | |
| Thrombin | >30 | | |

*All human except †rat and ‡sheep
Assays were repeated at least twice
## Extended Data Table 3 | In vivo pharmacokinetic analysis of KDU731 in mice, rats, monkeys, and calves

| Route | Parameter | Units | Mice | Rats | Rats TK* | Monkeys | Calves |
|-------|-----------|-------|------|------|----------|---------|--------|
|       | Dose      | mg/kg | 2.3  | 2.3  | 30       | 3       | 3      |
|       | C<sub>max</sub> | nM   | 406  | 161  | 19168    | 12       | 5      |
|       | AUC       | nM·h  | 2306 | 1844 | 93026(18x)| 1600    | 10     |
|       | t<sub>1/2</sub> | hours | 2.47 | 3.14 | -        | -        | 5<sup>a</sup> |
|       | F         | %     | 1.39 | 4.3  | 93026(18x)| 1600    | 4      |
|       | C<sub>max</sub> d.n | 177 | 70  | 638.9 | 93026(18x)| 1600    | 9      |
|       | AUC d.n   | 1003 | 802 | 3100.9 | 93026(18x)| 1600    | 12     |
| i.v.  | Dose      | mg/kg | 5    | 2.5  | -        | -        | 45.6   |
|       | V<sub>ss</sub> | L/kg | 1.12 | 2.15 | -        | -        | 45.6   |
|       | CL        | mL/min/kg | 16 | 12.4 | -        | -        | 45.6   |
|       | t<sub>1/2</sub> | hours | 1.06 | 3.3  | -        | -        | 45.6   |

Mice, Rats, Monkeys and Calves n = 4, 4, 3 and 7 respectively.

C<sub>max</sub>, maximum concentration achieved; AUC, area under curve (0-24 hours); t<sub>1/2</sub>, half-life; F, percentage oral bioavailability; C<sub>max</sub> d.n, dose-normalized C<sub>max</sub>; AUC d.n, dose-normalized AUC; V<sub>ss</sub>, steady state volume of distribution; CL, clearance; TK, toxicokinetic study.

p.o. denotes oral gavage formulated in 0.5% w/v methylcellulose and 0.5% w/v Polysorbate80 in water except for rats TK study.
i.v. denotes intravenous injection formulated in PEG300/DSW (3:1, v/v) for mice; Propylene Glycol / Tween 80 / Water (20:20:60, v/v) for rats and Methylpyrrolidone / PEG200 (10:90, v/v) for monkeys.

*Rats TK: day 1 TK analysis of KDU731 formulated in solid dispersion (SD) formulation as described in Methods section; numbers in parenthesis indicate the exposure multiples compared 21.3 mg per kg (body weight) suspension formulation.

Calves<sup>a</sup>, day 1, first dose PK data with KDU731 5 mg per kg (body weight) twice daily, AUC is 0.12 h.

Calves<sup>b</sup>, day 7, 13<sup>th</sup> dose PK data with KDU731 5 mg per kg (body weight) twice daily, AUC is 0.12 h.