

1. Further detail on assembly and screening of “NITD parasite box”. In previous work we have screened several large compound libraries against blood stage *Plasmodium falciparum* parasites and identified a number of novel active scaffolds and successfully advanced two new antimalarial drug candidates to the clinic³⁶. Our *P. falciparum* phenotypic screening efforts identified multiple new targets including the ATP-dependent sodium channel PfATP4⁹, phosphatidylinositol-4-OH-kinase¹², and lysyl-tRNA synthetase³⁷. While *Cryptosporidium* is an early branching member of the Apicomplexa and exhibits phylogenetic divergence from other members of the phylum, several druggable targets are conserved^{38,39}. To explore functional conservation of targets and pathways across parasites and to identify lead candidates against *Cryptosporidium*, we have assembled a “NITD parasite box” and screened it against *C. parvum* as described in the Supplementary Table 1. The “NITD parasite box” is a diversity set of 6220 non-cytotoxic (HepG2 SI > 20) and non-reactive compounds identified in a variety of anti-parasitic drug discovery projects among them *P. falciparum* phenotypic screening hits and compounds from 11 diverse scaffolds both active and inactive hits against *P. falciparum* and *Trypanosoma brucei* that inhibit the growth/viability of *C. parvum* in HCT-8 cells. During the screening the average infection rate of HCT-8 cells was 69% and the average z-factor of 0.74 compared to uninfected controls. We have observed a positive correlation between two replicates with an r^2 of the regression 0.831. The scatter plot of 6220 compounds with mean % inhibition is shown Fig 1a. We identified 154 new chemical entities (NCEs) that inhibited *C. parvum* infection by more than 3x SD from the mean of DMSO-treated controls, resulting in a 2.5 % hit rate. These 154 hits represented multiple compounds from Pf phenotypic screening hits and a few scaffolds active against PfPI(4)K, PfKRS etc. None of the *Trypanosoma* active hits showed activity against *C. parvum*. In this manuscript we focused our efforts on the pyrazolopyridine scaffold.

2. Structure-activity relationship analysis of pyrazolopyridines. Our previous phenotypic screening on *P. falciparum* efforts had identified imidazopyrazines¹². Further scaffold morphing of the imidazopyrazine core to improve drug like properties led to the identification of pyrazolopyridines¹⁴. We screened ~200 additional pyrazolopyridine analogs for *C. parvum* cellular activity. To understand the structure-activity relationship (SAR) we evaluated the impact of peripheral changes around the pyrazolopyridine core on *Cryptosporidium* activity (Table 1 and Supplementary Information below). In matched-pair comparisons with KDU731, replacing the primary amide on the phenyl ring with -CF₃ (KDU370) decreased Cp activity by 15-fold, suggesting the importance of primary amide. However, methyl substitution on the primary amide (KDZ464) is well tolerated, indicating a space for smaller lipophilic groups. Bulkier tertiary butyl substitution (LNN134) or N-demethylation (LMW740) of the tertiary amide significantly reduced *Cryptosporidium* activity indicating the importance of the size of the alkyl group and conformation of the tertiary amide for activity. Replacement of the pyridyl with a cyclohexyl (KEL204) significantly reduced the *Cryptosporidium* activity suggesting the importance of an aromatic ring and the planarity of KDU731. Overall the *Cryptosporidium* SAR of >200 derivatives follows that of *Plasmodium* and suggests that the mechanism of action of

pyrazolopyridines is conserved between these two parasites. Among the pyrazolopyridine analogs KDU731 showed the most promising cellular activity with a desirable pharmacokinetic and safety profile as described in further detail in the main text.

3. Further detail on calf efficacy study. Fifteen calves were enrolled in the study and 14 calves completed the study. Among the calves that completed the study, 7 calves were infected with *C. parvum* and treated with KDU731 (N101, N102, N103, N107, N108, N109 and N110), 6 calves were positive controls i.e., infected with *C. parvum*, untreated (N104, N111, N112, N113, N114 and N115), and 1 calf was a negative control i.e., uninfected with *C. parvum*, untreated (N104). Calf N105 died acutely within 72 hours of enrollment, prior to initiation of treatment with KDU731, and was excluded from analysis. The remaining enrolled calves had a mean serum total protein (g/dl) of 5.6 ± 0.5 (95 % CI 5.3–5.8) (range 5 – 6.3). The mean was higher in the positive control group ($P = 0.02$), however, each group attained adequate passive transfer of colostral antibody (>5.0 g/dl). The negative control calf did not shed oocysts or have clinical signs consistent with cryptosporidiosis. The 13 calves challenged with *C. parvum* oocysts all shed oocysts in their stool and developed clinical signs consistent with cryptosporidiosis. Infection followed the anticipated pattern of fecal shedding.

Calves treated with KDU731 shed significantly fewer oocysts ($P < 0.0001$) (Fig. 3b). The mean log oocysts counted per gram of fecal dry matter during the 7 day treatment period was 3.5 ± 1.1 for the positive control calves and 2.3 ± 1.2 for the calves treated with KDU731. During the first 2 days of treatment, there was not a significant difference in fecal oocyst shedding between treated and control calves. By the third day of treatment, calves receiving KDU731 shed significantly fewer oocysts ($P < 0.0001$) and experienced a 2-log reduction in oocyst shedding.

Treated calves also experienced substantial improvement in severity of clinical illness in comparison to untreated controls (Fig 3c, 3d and Extended data Figure 7). During the treatment period, the KDU731-treated calves had a median fecal consistency score of 2 and a median dehydration score of 1. During the equivalent phase of infection, the untreated controls had a median fecal consistency of 3 and a median dehydration score of 2. The KDU731-treated calves had significantly fewer days of severe diarrhea ($P = 0.0004$) and severe dehydration ($P < 0.0001$) (Fig 3c, 3d and Extended data Figure 7). By the end of the second day of treatment, all calves returned to a hydration score of 1 and maintained this for the duration of the study (Extended data Figure 7).

Calf N104 developed seizures on day 4 of enrollment. Calf N104 was an untreated positive control calf. Therefore, the seizure activity was not attributed to KDU731. Calf N104 remained in the study under close observation and was treated with a single dose of oral diazepam (10mg) (IVAX Pharmaceuticals, Miami, FL) to control the seizure activity. Seizures subsided and the calf remained enrolled in the study. In an effort not to cause stimulation and induce seizures, the frequency of sampling was reduced for this calf. The necropsy of calf N104 did not reveal a cause for the seizure activity. It is likely the seizures were attributable to a congenital anomaly.

KDU731 treated calves N108, N109, and N110 developed infections at the site of the intravenous catheters resulting in sepsis and joint infection. The calves were not treated with antibiotics due to concerns of possible drug-drug interactions. Calves were treated with a non-

steroidal anti-inflammatory for pain. Calves N108 and N109 completed treatment with KDU731 and exited the study early. Upon development of joint infection, calf N108 was removed from PK sampling. Calf N109 exited the study prior to the cessation of fecal oocyst shedding. There were no significant differences in fecal oocyst shedding (two-tailed $P = 0.9$), fecal consistency score (two-tailed $P = 0.6$), or dehydration score (two-tailed $P = 0.8$) between KDU731-treated calves that developed a co-morbidity and those that did not. All calves were in excellent post mortem state with adequate amounts of abdominal adipose tissue except for the typical cryptosporidiosis condition. There is no evidence of toxicity in association with treatment in the skeletal muscle (diaphragm, tongue), lymphoid tissue (ileocecal lymph node, spleen), liver, kidney and heart.

Calf Clinical Illness Rubric. Clinical illness is evaluated on an ordinal scale every 12 h.

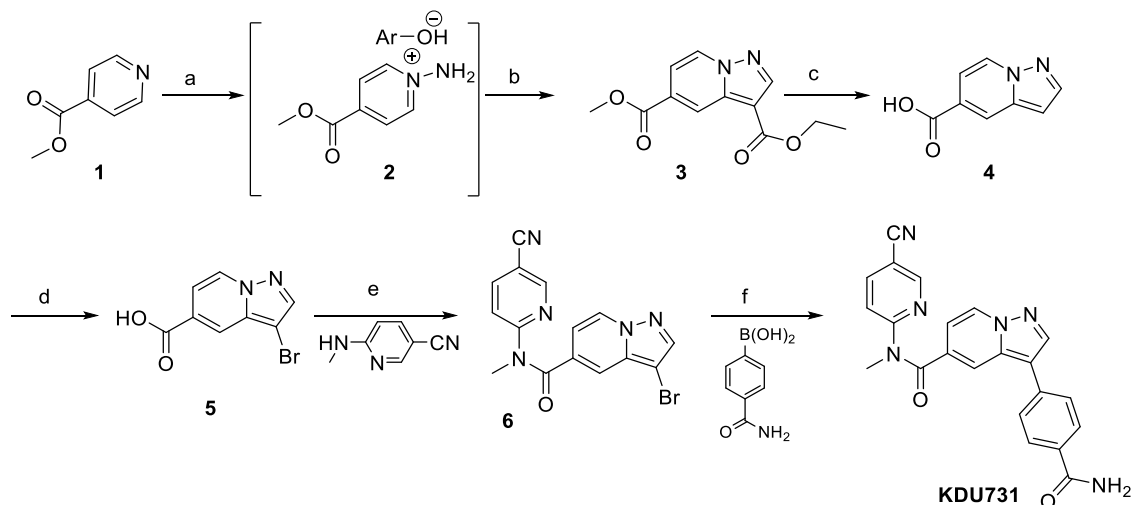
Score	Appetite	Mentation	Fecal Consistency	Hydration
1	Normal ; Hungry, eats greedily, often twitching its tail as it eats, strong suckle reflex is present.	Normal ; Alert and watches the caretakers, reacts when caretakers enter the stall. May stretch when standing to rise or meet caretakers.	Normal ; Feces retain form. The feces may be pasty but do not flow across a surface.	Normal to 5% Dehydration ; Euhydrated or $\leq 5\%$ dehydrated. Normal stool or mild-moderate diarrhea, appetite and mentation score =1, and skin tenting < 2 seconds.
2	Slight to moderate ; Eats with some coaxing but not aggressively, suckle reflex is present but may not be vigorous.	Mildly to moderately depressed ; Pays some attention to caretakers, and is reluctant rise but will rise with coaxing.	Mild to moderate diarrhea ; Form is a puddle, not a patty. Sufficient water content to slowly flow across or down a surface leaving some material adherent to the surface.	6-8% Dehydration ; Moderate-severe diarrhea, skin tenting 2-6 seconds, mild-moderate sunken eyes, mild-moderate depression, mentation score =2, suckle reflex is present but not vigorous, appetite score = 2.
3	Abnormal ; Refuses to eat even when coaxed, suckle reflex is weak or absent.	Severely depressed ; Pays no attention to caretakers, and will not rise even with coaxing. May or may not be moribund.	Severe diarrhea ; Watery feces, easily flow across or down a surface leaving little to no adherent material. Calf may have very watery feces followed by some solid material and still have severe diarrhea.	> 8% Dehydration ; Severe diarrhea, skin tenting > 6 seconds, fully sunken eyes, recumbent, may not be able stand, moderate to severe depression, mentation score ≥ 2 , suckle reflex is weak or absent, appetite score = 3.

4. Chemical synthesis. Unless otherwise noted, materials were obtained from commercial suppliers, of the best commercially available grade, and were used without purification. Removal of solvent under reduced pressure refers to distillation using Büchi rotary evaporator attached to a vacuum pump. ^1H NMR spectra were determined on a Bruker Ultrashield 400 MHz spectrometer. Proton resonances are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). ^1H NMR data are reported as multiplicity (s - singlet, d - doublet, t - triplet, q - quartet, quint - quintet, sept - septet, dd - doublet of doublets, dt - doublet of triplets, bs - broad singlet), number of protons and coupling constant in Hertz. Flash chromatography was performed either by CombiFlash® (Separation system Sg. 100c, ISCO) or using silica gel (Merck Kieselgel 60, 230-400 mesh). LC-MS analysis were performed using Waters Acquity UPLC with Quattro-micro detector; Waters Acquity BEH C18, 1.7 μm , 2.1 X 50 mm column; gradient of 90:10 H_2O (0.025% TFA): CH₃ CN (0.025% TFA) hold for 0.5 min and to 10:90 H_2O (0.025% TFA):CH₃ CN (0.025% TFA) in 3.0 min and hold for 2.0 min with flow rate of

0.4 mL/min and HPLC purity were performed using Waters Acquity UPLC with PDA detector; Waters Acquity BEH C18, 1.7 μ m, 2.1 X 100 mm column; gradient of 70:30 H₂O (0.025% TFA): CH₃CN (0.025% TFA) to 20:80 H₂O (0.025% TFA):CH₃CN (0.025% TFA) in 4 min and hold for 2 min with flow rate of 0.3 mL/min. All the synthesized compounds were with purity >95% based on ¹H NMR and HPLC analysis.

4.1 Synthesis of KDU731:

KDU731 was synthesized in five steps starting from commercially available methyl isonicotinate (CAS Number 2459-09-8).



Reagents and conditions: (a) *O*-(3,4-dinitrophenyl)hydroxylamine, DMF, 40 °C, 16 h; (b) ethyl propiolate, K₂CO₃, DMF, room temperature, 16 h, 74% (over twosteps); (c) 40% H₂SO₄, 100 °C, 16 h, 91%; (d) *N*-bromosuccinimide (NBS), DMF, 0 °C to rt, 30 min, 74%; (e) oxalyl dichloride, CH₂Cl₂, DMF, (cat), 0 °C, 60 min, followed by amine, DIPEA, 0 °C to room temperature, 18 h, 67%; (f) Pd₂(dba)₃•CHCl₃, X-phos, NaHCO₃, ACN/H₂O (9:1), 100 °C, 3 h, 51%.

4.1.1 Synthesis of intermediate (3), 3-ethyl 5-methyl pyrazolo[1, 5-a]pyridine-3, 5-dicarboxylate:

To a solution of methyl isonicotinate **1** (500 μ L, 4.23 mmol, 1.0 equiv.) in 2 mL DMF was added *O*-(2, 4-dinitrophenyl)hydroxylamine (1 g, 5.02 mmol, 1.2 equiv.) and the solution was allowed to stir overnight at 40 °C. The mixture was allowed to cool to room temperature before being diluted with 10 mL DMF, then treated with ethyl propiolate (536 μ L, 5.29 mmol, 1.25 equiv.) and potassium carbonate (877 mg, 6.35 mmol, 1.5 equiv.). The mixture was allowed to stir overnight at room temperature. The solvent was removed under reduced pressure and the residue was diluted with water (20 mL). The aqueous layer was extracted with EtOAc (50 mL X 5). The combined EtOAc extracts were washed with water (1 X 50 mL), brine (1 X 50 mL), then dried on MgSO₄ and the organic layer was evaporated under reduced pressure. The crude product was purified by silica gel chromatography with hexanes/EtOAc to give 0.78 g (74%

yield) of **3**. ^1H NMR (400 MHz, CDCl_3): δ 8.86 (dd, $J = 1.8, 0.9$ Hz, 1H), 8.55 (dd, $J = 7.2, 0.9$ Hz, 1H), 8.47 (s, 1H), 7.52 (dd, $J = 7.2, 1.8$ Hz, 1H), 4.42 (q, $J = 7.1$ Hz, 2H), 3.99 (s, 3H), 1.44 (t, $J = 7.1$ Hz, 3H). ESI-LC/MS: $m/z = 249.0$ ($\text{M}+\text{H}$) $^+$.

4.1.2 Synthesis of intermediate (4), Pyrazolo[1, 5-a] pyridine-5-carboxylic acid:

A suspension of diester **3** (8.5 g, 34.2 mmol, 1.0 equiv.) in 150 mL of 40% H_2SO_4 was allowed to heat at 100 °C for overnight in a septum-capped vial fitted with a needle outlet to an empty balloon to accommodate the gas evolution. The resulting solution was allowed to cool to rt, then placed in a cold water bath before bringing to approximately pH = 5 with 6N NaOH. During this pH adjustment, the acid precipitated and was isolated by filtration. The solid was washed with water and dried under high vacuum overnight to provide 5.06 g (91%) of acid **4**. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 12.5 (br s, 1H), 8.74 (d, $J = 7.3$ Hz, 1H), 8.34 (s, 1H), 8.11 (d, $J = 2.3$ Hz, 1H), 7.25 (dd, $J = 7.3, 1.9$ Hz, 1H), 6.89 (dd, $J = 2.3, 0.8$ Hz, 1H). ESI-LC/MS: $m/z = 163.2$ ($\text{M}+\text{H}$) $^+$.

4.1.3 Synthesis of intermediate (5), 3-Bromopyrazolo[1,5-a]pyridine-5-carboxylic acid:

To a stirred solution of *pyrazolo[1,5-a]pyridine-5-carboxylic acid* **4** (30.0 g, 0.18 mol) in DMF (150 mL) was added NBS (36.24 g, 0.20 mol) at 0 °C in small portions over a period of 30 min. The reaction mixture was allowed to rt and stirred for 30 min. The reaction mixture was quenched in to cold water (1 L), stirred vigorously for 30 min. The precipitated solid was washed with water (500 mL), dried under vacuum. The crude compound was triturated with ethyl acetate (200 mL), filtered the solid, washed with ethyl acetate (100 mL) and dried under vacuum to afford 33.0 g (74%) of *3-bromopyrazolo[1,5-a]pyridine-5-carboxylic acid* **5** as an off white solid. ESI-LC/MS: $m/z = 240.90$ ($\text{M}+\text{H}$) $^+$ & 242.94 [($\text{M}+2$)+ H] $^+$.

4.1.4 Synthesis of intermediate (6), 3-bromo-N-(5-cyanopyridin-2-yl)-N-methylpyrazolo[1,5-a]pyridine-5-carboxamide:

To a solution of 3-bromopyrazolo[1,5-a]pyridine-5-carboxylic acid **5** (7.0 g, 29 mmol) in dichloromethane (70 mL) was added oxalyl chloride (2.8 mL, 31.9 mmol) followed by catalytic amount dimethylformamide (0.214 mL) at 0 °C and stirred at room temperature for 60 min. The resultant volatiles were distilled-off under reduced pressure. The resulting crude acid chloride was dissolved in dichloromethane (50 mL) was added to a solution of 6-(methylamino)nicotinonitrile (3.48 g, 26.1 mmol) and DIPEA (30.4 mL, 174 mmol) in dichloromethane (25 mL). The reaction mixture was stirred for 18 h at room temperature and diluted with dichloromethane (150 mL). The organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and concentrated. The crude compound was purified by column chromatography over silica-gel (100-200 mesh) using hexanes/EtOAc to afford 7.0 g (63%) of *3-bromo-N-(5-cyanopyridin-2-yl)-N-methylpyrazolo[1,5-a]pyridine-5-carboxamide* **6** as a white solid. ^1H NMR (400 MHz, CDCl_3): δ 8.67 (s, 1H), 8.34 (d, $J = 8.0$ Hz, 1H), 7.96 (s, 1H), 7.81 (dt, $J = 8.7, 2.0$ Hz, 1H), 7.66 (s, 1H), 7.36 (d, $J = 8.5$ Hz, 1H), 6.74 (dd, $J = 7.3, 2.1$ Hz, 1H), 3.62 (s, 3H). ESI-LC/MS: $m/z = 358.9$ [($\text{M}+2$)+ H].

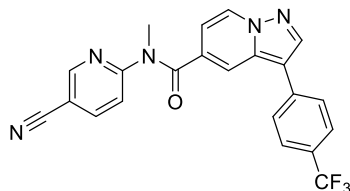
4.1.5 Synthesis of KDU731, 3-(4-carbamoylphenyl)-N-(5-cyanopyridin-2-yl)-N-methylpyrazolo[1,5-a]pyridine-5-carboxamide:

A solution of 3-bromo-N-(5-cyanopyridin-2-yl)-N-methylpyrazolo[1,5-a]pyridine-5-carboxamide **6** (12.5 g, 35.09 mmol), 4-(N-methylaminocarbonyl)phenylboronic acid (8.68 g, 52.6 mmol) and NaHCO₃ (5.89 g, 70.1 mmol) in acetonitrile: water (9: 1) (130 mL) was degassed with argon for about 30 min. To this mixture were added Pd₂(dba)₃•CHCl₃ adduct (3.63 g, 3.509 mmol) and X-phos (4.87 g, 10.52 mmol) under argon atmosphere. The resulting reaction mixture was maintained at 100 °C for 3 h then allowed to room temperature and filtered through celite. The filtrate was partitioned between water (500 mL) and ethyl acetate (500 mL). The ethyl acetate layer was washed with brine, dried over anhydrous Na₂SO₄ and concentrated. This crude compound was purified by column chromatography over silica gel (100-200 mesh) using a solvent gradient of 3.4% methanol in dichloromethane to obtain 7.1 g (51%) of 3-(4-carbamoylphenyl)-N-(5-cyanopyridin-2-yl)-N-methylpyrazolo[1,5-a]pyridine-5-carboxamide **NVC-KDU731** as a yellow color solid. ¹H NMR (400 MHz, DMSO-d₆): δ 8.87 (dd, *J* = 2.3, 0.8 Hz, 1H), 8.72 (dd, *J* = 7.2, 0.9 Hz, 1H), 8.53 (s, 1H), 8.26 (dd, *J* = 8.6, 2.3 Hz, 1H), 8.01 (s, 1H), 7.99-7.94 (m, 3H), 7.66-7.59 (m, 3H), 7.37 (s, 1H), 6.86 (dd, *J* = 7.2, 1.8 Hz, 1H), 3.54 (s, 3H); ESI-MS: *m/z* 398(M+H)⁺.

4.2 Other pyrazolopyridine analogs:

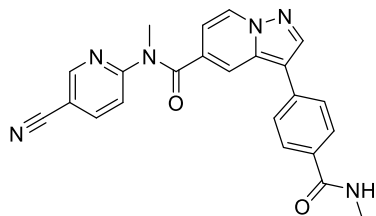
Other pyrazolopyridine analogs were prepared by using the above synthetic scheme with appropriate starting materials. The structures and compounds analytical data given below.

4.2.1 KDU370 N-(5-cyanopyridin-2-yl)-N-methyl-3-(4-(trifluoromethyl)phenyl)pyrazolo[1,5-a]pyridine-5-carboxamide



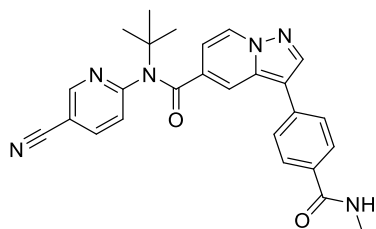
¹H NMR (400 MHz, CDCl₃): δ 8.67 (dd, *J* = 2.3, 0.8 Hz, 1H), 8.41 (dd, *J* = 7.2, 0.7 Hz, 1H), 8.21 (s, 1H), 7.98 (m, 1H), 7.80 (dd, *J* = 8.6, 2.3 Hz, 1H), 7.69 (d, *J* = 8.2 Hz, 2H), 7.59 (d, *J* = 8.1 Hz, 2H), 7.35 (dd, *J* = 8.7, 0.8 Hz, 1H), 6.73 (dd, *J* = 7.2, 1.9 Hz, 1H), 3.62 (s, 3H). ESI-MS: (*m/z*) 422. 1 [M+H]⁺.

4.2.2 KDZ464: N-(5-cyanopyridin-2-yl)-N-methyl-3-(4-(methylcarbamoyl)phenyl)pyrazolo[1,5-a]pyridine-5-carboxamide



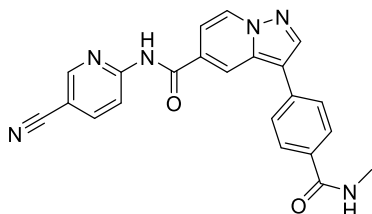
^1H NMR (400 MHz, DMSO- d_6): δ 8.85 (s, 1H), 8.71 (d, J = 6.8 Hz, 1H), 8.52 (s, 1H), 8.46 (br s, 1H), 8.25 (d, J = 8.4 Hz, 1H), 7.98 (s, 1H), 7.96 (d, J = 8.0 Hz, 2H), 7.64-7.61 (m, 3H), 6.84 (d, J = 7.2 Hz, 1H), 3.53 (s, 3H), 2.81 (d, J = 4.0 Hz, 3H). ESI-LC/MS: m/z = 411.30 ($M+H$) $^+$.

4.2.3 LNN134: N-(tert-butyl)-N-(5-cyanopyridin-2-yl)-3-(4-(methylcarbamoyl)phenyl)pyrazolo[1,5-a]pyridine-5-carboxamide



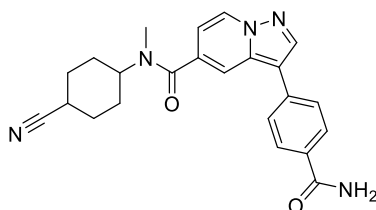
^1H NMR (400 MHz, DMSO- d_6): δ 8.96 (s, 1H), 8.58 (d, J = 6.6 Hz, 1H), 8.48 (s, 1H), 8.42 (s, 1H), 8.29 (d, J = 7.9 Hz, 1H), 7.97 (d, J = 7.0 Hz, 2H), 7.77 (d, J = 7.4 Hz, 1H), 7.66 (s, 1H), 7.58 (d, J = 7.5 Hz, 2H), 6.76 (d, J = 7.4 Hz, 1H), 2.82 (s, 3H), 1.47 (s, 9H). ESI-LC/MS: m/z = 453.19 ($M+H$) $^+$.

4.2.4 LMW740: N-(5-cyanopyridin-2-yl)-3-(4-(methylcarbamoyl)phenyl)pyrazolo[1,5-a]pyridine-5-carboxamide



^1H NMR (400 MHz, DMSO- d_6): δ 11.81 (s, 1H), 8.91 (s, 1H), 8.86 (d, J = 7.0 Hz, 1H), 8.73 (s, 1H), 8.59 (s, 1H), 8.46-8.52 (m, 1H), 8.33-8.40 (m, 2H), 7.93-7.99 (m, 4H), 7.41 (d, J = 7.0 Hz, 1H), 2.81 (d, J = 4.4 Hz, 3H). ESI-LC/MS: m/z 397.13 ($M+H$) $^+$.

4.2.5 KEL204: 3-(4-carbamoylphenyl)-N-(4-cyanocyclohexyl)-N-methylpyrazolo[1,5-a]pyridine-5-carboxamide



^1H NMR (400 MHz, CD $_3$ OD): δ 8.68- 8.66 (m, 1H), 8.38 (s, 1H), 7.99-7.97 (m, 3H), 7.78-7.76 (m, 2H), 7.0- 6.93 (m, 1H), 4.57-4.40 (m, 0.5H), 3.70-3.59 (m, 0.5H), 3.19-2.90 (m, 3.5H), 2.63- 2.50 (m, 0.5H), 2.30-1.63 (m, 7H), 1.59-1.40 (m, 1H). ESI-LC/MS: m/z 402.14 ($M+H$) $^+$.

5. CpPI4K-S871L and CpPI4K- H1037Y mutants' enzyme analysis. The molecular target PI(4)K in *P. falciparum* has been validated by generating resistant mutants¹². These mutants showed a copy number variation and in some cases point-mutations in the molecular target (PfPI4K H1484Y and PfPI4K S1320L). To evaluate the role of homologous CpPI(4)K residues (CpPI4K-H1037Y and CpPI4K-S871L) in interactions with pyrazolopyridines, we engineered mutant forms of the CpPI4K enzyme. KDU731 inhibited CpPI4K- H1037Y and CpPI4K-S871L enzymes with an IC₅₀ of 11 ± 1.9 nM and 8.5 ± 0.2 nM respectively, compared to 13.7 ± 6.5 nM for the wild type CpPI(4)K in the same experiment. Thus, these mutations do not significantly shift susceptibility of the *C. parvum* enzyme to KDU731 suggesting subtle differences between *Cryptosporidium* and *Plasmodium* in the ligand binding pocket.

6. Rat toxicology study:

Suspension formulation with limited systemic exposure has been used in both mouse and neonatal calf models. Though GI exposure is good enough for efficacy, ideally we need systemic exposure to demonstrate safety. KDU731 in solid dispersion (SD) significantly enhanced systemic exposure in rats compared to suspension formulation (Extended Data Table 3). Hence, SD formulation was used to demonstrate *in vivo* safety in 2 weeks rat toxicology studies with KDU731. All rats were monitored for changes in body weight, food consumption and organs weight; and clinical and histopathological analyses were undertaken. All rats tolerated oral administration of KDU731 (30 and 100 mg/kg) daily for 14 days with no mortality and no drug-related changes food consumption or body weight gain were observed. Toxicokinetic studies revealed that no accumulation of KDU731 was observed after repeated administration for 14 days. No significant differences in hematology parameters were observed between the KDU731 treated and control groups with the exception of a minor increase in red blood cells (RBC) count. A slight elevation in cholesterol and phosphate ions and a decrease in glucose levels were observed in 100 mg/kg KDU731 treated group. No other significant changes in the blood chemistry parameters were observed. Overall, clinical chemistry and hematology findings at 100 mg/kg were minor and monitorable. Minimal to slight pathological changes were detected in both the vehicle and high-dose groups, and no obvious pathological changes directly related to the test agent were found. Overall, under the study conditions KDU731 at 30 mg/kg/day is considered as the NOEL (no observable effect level).

REFERENCES

36. Diagana, T. T. Supporting malaria elimination with 21st century antimalarial agent drug discovery. *Drug discovery today* **20**, 1265-1270, doi:10.1016/j.drudis.2015.06.009 (2015).
37. Hoepfner, D. *et al.* Selective and specific inhibition of the plasmodium falciparum lysyl-tRNA synthetase by the fungal secondary metabolite cladosporin. *Cell host & microbe* **11**, 654-663, doi:10.1016/j.chom.2012.04.015 (2012).
38. Abrahamsen, M. S. *et al.* Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. *Science* **304**, 441-445, doi:10.1126/science.1094786 (2004).
39. Xu, P. *et al.* The genome of *Cryptosporidium hominis*. *Nature* **431**, 1107-1112, doi:10.1038/nature02977 (2004).

Supplementary Table 1. Small molecule screening data

Category	Parameter	Description
Assay	Type of assay	Immunofluorescence <i>Cryptosporidium</i> HCT-8 infection assay
	Target	<i>Cryptosporidium parvum</i> growth inhibition
	Primary measurement	High Content Imaging
	Key reagents	Fluorescein-labelled <i>Vicia villosa</i> lectin, Hoechst, RPMI medium
	Assay protocol	HCT-8 cells were seeded into 96-well, black, flat and clear-bottom plates. Following overnight incubation, HCT-8 cells were infected in a serum-free RPMI medium with excysted <i>C. parvum</i> oocysts. Three hours later, the cells were washed to remove oocysts and extracellular sporozoites and infected cells were incubated in RPMI medium with 10% serum. Compounds were added to infected cells to a final concentration of 5 μ M in duplicate and plates were incubated for 48 hours prior to fixation and stained using fluorescein-labelled <i>Vicia villosa</i> lectin, a parasite specific lectin and Hoechst for enumeration of host cell nuclei. Plates were analyzed using a high content imaging system to determine percent infected cells; percent inhibition was calculated relative to vehicle treated control wells. HCT-8 cell numbers were monitored to identify potentially cytotoxic compounds.
	Additional comments	Single-point screening was carried out with two different batches of Oocysts.
Library	Library size	6220 compounds
	Library composition	This library consists of <i>P. falciparum</i> phenotypic screening hits and compounds from 11 diverse scaffolds both active and inactive hits against <i>P. falciparum</i> and <i>Trypanosoma brucei</i> .
	Source	NITD compound collection
	Additional comments	Chemical library has non-cytotoxic (HepG2 SI > 20) and non-reactive compounds.
Screen	Format	96-well black/clear bottom plates
	Concentration(s) tested	5 μ M in technical replicates
	Plate controls	DMSO as negative control and Nitazoxanide (5 μ M) as positive control.
	Reagent/ compound dispensing system	Using compound dispenser, final DMSO concentration is 0.5%.
	Detection instrument and software	Plates are scanned on a high content system and the percent of infected cells are determined
	Assay validation/QC	The average infection rate of HCT-8 cells was 69% & the average z-factor 0.74 compared to uninfected control.
	Correction factors	-
	Normalization	Infection compared to DMSO control
	Additional comments	Single-point screening was carried out in ~160 assay plates over a few weeks. Each assay plate has column 1 and 12 are used for controls.
Post-HTS analysis	Hit criteria	More than 3x SD from the mean of DMSO treated controls.
	Hit rate	2.5% (154 hits)
	Additional assay(s)	Hits were reconfirmed in a 10-point dose-response HCl assay in 384-well flat black clear-bottom plates using an Opera QEHS (PerkinElmer™) Imaging system.
	Confirmation of hit purity and structure	Purity >85% and compound structure verified by analytical chemistry methods.
	Additional comments	EC50 were reconfirmed against both <i>C. parvum</i> and <i>C. hominis</i> in High Content Imaging (HCI) and a cytopathic effect (CPE) based assay as described in the Methods. Validated hits were also evaluated in HepG2 cytotoxicity assay.