SUPPORTING INFORMATION

Repositioning of a diaminothiazole series confirmed to target the cyclin-dependent kinase CRK12 for use in the treatment of African animal trypanosomiasis

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1. Parasitology

1.1 In vitro drug sensitivity assays - Trypanosoma brucei brucei

Drug sensitivity assays were carried out with bloodstream-form (BSF) T. b. brucei ‘single marker’ S427 cells grown at 37°C with 5% CO₂ in HMI-9T medium,¹ as previously described.²

1.2 In vitro drug sensitivity assays - Trypanosoma congolense

EC₅₀ values for test compounds against T. congolense (IL3000 strain) were conducted as previously described³ with minor modifications.⁴ Briefly, trypanosome cell densities were determined using a CASY cell counter (Schärfe) and diluted to a seeding density of 2 x 10⁵ trypanosomes/mL. BSF trypanosomes were grown in HMI-9 (IMDM) media supplemented with 20% (v/v) bovine serum.¹ Test compounds were resuspended to a final concentration of 10 mg/mL in DMSO. Assays plates were incubated at 34 °C with 5 % CO₂, for a total of 69 h, prior to addition of 10 µL of Resazurin (12.5 mg in 100 mL phosphate buffered saline, Aldrich) into each well, followed by a further 3 h incubation. Absorbance was read using a SpectraMax (Gemini XS) fluorescence reader at an excitation wavelength of 536 nm and an emission wavelength of 588 nm. EC₅₀ values were determined using SOFTmax Pro 5.2 analysis software. All experiments were conducted with technical duplicates, and at least three biological replicates.

1.3 Ex vivo drug sensitivity assays - Trypanosoma vivax

Since T. vivax cannot be cultured axenically, BSF trypanosomes were harvested from an infected mouse with high parasitemia via cardiac puncture and used directly in ex vivo assays. EC₅₀ values were determined against T. vivax (STIB 719/ ILRAD 560) as previously described³, with minor modifications. Briefly, cell densities were determined using a Neubauer chamber and then diluted to a seeding density of 4 x 10⁵ trypanosomes / mL. BSF trypanosomes were cultured in HMI-9 (IMDM) media containing 20% (v/v) bovine serum. Assay plates were incubated at 37 °C with 5 % CO₂, for a total of 45 h prior to addition 10 µL of Resazurin (12.5 mg in 100 mL phosphate buffered saline, Aldrich) into each well, followed by a further 3 h incubation. Absorbance was read using a SpectraMax (Gemini XS) fluorescence reader at an excitation wavelength of 536 nm and an emission wavelength of 588 nm. EC₅₀ values were determined using SOFTmax Pro 5.2 analysis software.
software. All experiments were conducted with technical duplicates and at least three biological replicates.

1.4 Cytotoxicity assays – Rat skeletal myoblasts (L6)
The cytotoxicity of test compounds was evaluated using a rat skeletal myoblast (L6) cell line, as previously described, but with minor modifications. Cells were grown in RPMI media supplemented with 10% (v/v) fetal calf serum. L6 cells at a seeding density of 2 x 10^4 cells/mL were incubated in microtiter plates overnight at 37 °C and 5 % CO₂ until adherent. The following day, test compounds were added to the plates (3-fold serial dilutions), prior to incubation for 69 h. Following incubation, 10 µL of Resazurin (12.5 mg in 100 mL phosphate buffered saline, Aldrich) was added to each well and the plates were incubated for a further 3 h. The plates were read using a SpectraMax (Gemini XS) fluorescence reader at an excitation wavelength of 536 nm and an emission wavelength of 588 nm. EC₅₀ values were determined using SOFTmax Pro 5.2 analysis software. All experiments were conducted as technical replicates, with at least three biological replicates.

2. Drug metabolism and pharmacokinetics

2.1 Solubility
The aqueous solubility of test compounds was measured using laser nephelometry. Compounds were subject to serial dilution from 10 mM to 0.5 mM in DMSO. Aliquot were then mixed with MilliQ water to obtain an aqueous dilution plate with a final concentration range of 250 – 12 µM. The final DMSO concentration in these plates was 2.5%. Triplicate aliquots were transferred to a flat-bottomed polystyrene plate which was immediately read on a NEPHELOstar nephelometer (BMG Lab Technologies). The laser scatter caused by insoluble particulates (relative nephelometry units, RNU) was plotted against compound concentration using a segmental regression fit, with the point of inflection being quoted as the compound’s aqueous solubility (µM).

Solubility of compound 1 was measured by a different method. Here, compound 1 was dissolved in DMSO to give a 10 mM solution and solubility test samples prepared by adding a volume (5 µL) of this stock solution to phosphate buffered saline, pH 7.4 (195 µL, Sigma-Aldrich). This
solution was then mixed for 24 h (rotary mixing, 900 rpm, 25°C) excluding light. After mixing, test samples were filtered to remove any undissolved material using a proprietary filter (Millipore Multiscreen HTS filter, 96-well format). Samples were drawn through the filter using a vacuum. The resulting filtrate was analysed for dissolved compound 1 using a truncated UHPLC methodology. A Shimadzu Nexera X2 UHPLC system was used, with a reversed-phase column and a simple formic acid gradient elution. The UHPLC parameters are shown below (Table S1):

### Table S1: UHPLC Parameters

| Parameter                  | Value                                                      |
|----------------------------|------------------------------------------------------------|
| **Mobile phase component A** | HPLC water plus 0.1% formic acid                          |
| **Mobile phase component B** | HPLC acetonitrile plus 0.1% formic acid                    |
| **Flow rate:**             | 0.6 mL/min                                                 |
| **Gradient program:**      | Initial: 98% A, 2% B                                        |
|                            | At 1.2 min: 2% A, 98% B                                    |
|                            | At 2.0 min: 2% A, 98% B                                    |
|                            | Re-equilibration time: 0.6 min                             |
| **Autosampler temperature:** | 25°C                                                       |
| **Column:**                | Hypersil Gold, C18 1.9 µm, 50 × 2.1 mm                     |
| **Column temperature:**    | 50°C                                                       |
| **Detector wavelength:**   | 254 nm                                                     |
| **Bandwidth:**             | 4 nm                                                       |

A calibration solution was prepared as follows: The same 10 mM solution used to prepare the solubility test sample was diluted in DMSO to give a 500 µM solution. This solution was further diluted with 50:50 acetonitrile:water to give a final 50 µM solution. Aliquots (0.2, 2.0 and 5.0 µL) of this 50 µM solution were injected onto the UHPLC system and the areas of the resultant peaks integrated to produce a calibration line. Aliquots of the test sample filtrate (0.4 and 5.0 µL) were then injected onto the UHPLC system and the resultant peak areas for any peaks corresponding to the test compound determined and quantified using the calibration line. Although this is also a “kinetic”
solubility method, the long shaking times appear to give solubility values that more closely reflect those made from solid samples (unpublished in-house data).

2.2 Microsomal stability studies

(a) Mouse liver microsome stability. These methods have been previously reported.6

(b) Bovine liver microsome stability. Test compounds (0.5 µM) were incubated with 0.5 mg/mL bovine liver microsomes (Xenotech LLC™) in 50 mM potassium phosphate buffer, pH7.4 and the reaction initiated upon addition of excess NADPH (8 mg/mL in 50 mM potassium phosphate buffer, pH7.4). Aliquots (50 µL) of the incubation mixture were taken at time zero, 3, 6, 9, 15 and 30 min then mixed with acetonitrile (100 µL) to stop the reaction. An internal standard was added to all samples, prior to centrifugation (10 min, 1,665 g, RT) to sediment precipitated protein. Reaction plates were then sealed prior to UPLCMSMS analysis using a Quattro Premier XE (Waters corporation, USA).

XLfit (IDBS, UK) was used to calculate the exponential decay and consequently the rate constant (k) from the ratio of peak area of test compound to internal standard at each timepoint. The rate of intrinsic clearance (CLi) of each test compound was then calculated using the following calculation:

\[ \text{CLi (mL/min/g liver)} = k \times V \times \text{microsomal protein yield} \]

Where V (mL/mg protein) is the incubation volume/ mg protein added and microsomal protein yield is taken as 52.5 mg protein/ g of liver. Verapamil (0.5 µM) was used as a positive control to confirm acceptable assay performance.

2.3 Hepatocyte stability studies

(a) Mouse hepatocyte stability studies. Cryopreserved vials of mouse cryopreserved hepatocytes, supplied by Life Technologies, were thawed according to manufacturer’s instructions and cells resuspended in Williams Medium E (WME) containing cell maintenance supplement pack (CM4000, Life Technologies). Hepatocytes were incubated in suspension (0.5 million cells/mL) in 48 well non-
collagen coated cell culture plates for 10 minutes at 37°C, 5% CO₂. Upon addition of an equal volume of supplemented WME containing 1 µM test compound, an aliquot of incubation solution was removed to acetonitrile containing internal standard (final concentration 0.5 µM test compound and a cell density of 0.25 million cells/mL). Similarly, aliquots were removed at 3, 6, 9, 15, 30, 45, 60, 90 and 120 min. 100 µL of 80:20 water:acetonitrile was added to all samples and the analysis plate was centrifuged (10 min, 1,665 g, RT) prior to injection and analysis of samples by UPLC-MS/MS. The response (area ratio of test compound relative to internal standard) was plotted against time using an exponential decay model and rate of disappearance calculated. Hepatocyte CLint (mL/min/10⁶ cells) was scaled to in vivo CLint (mL/min/g Liver) using the hepato-cellularity scaling factor of 120 x 10⁶ cells/g of liver.

(b) Bovine hepatocyte stability. Freshly isolated female bovine (Prim’Holstein) hepatocytes were supplied by Biopredic International™. Cells were resuspended in Williams Medium E (WME) containing cell maintenance supplement pack (CM4000, Life Technologies). Hepatocytes were incubated in suspension (5 x 10⁵ cells/mL) in 48 well non-collagen coated cell culture plates for 10 min at 37°C. Upon addition of an equal volume of supplemented WME containing 1 µM test compound. Aliquots of incubation solution were removed and added to acetonitrile containing internal standard (final concentration 0.5 µM test compound and a cell density of 7 x 10⁵ cells/mL). Similarly, aliquots were removed at 10, 20, 30, 45, 60, 75, 90, 105 and 120 min. 100 µL of 80:20 water:acetonitrile was added to all samples and the analysis plate was centrifuged (10 min, 1,665 g, RT) prior to injection and analysis of samples by UPLC-MS/MS. The response (area ratio of test compound to internal standard) was plotted against time using an exponential decay model and rate of disappearance calculated. Hepatocyte CLint (mL/min/10⁶ cells) was scaled to in vivo CLint (mL/min/g Liver) using the hepato-cellularity scaling factor of 120 x 10⁶ cells/g of liver.
2.4 Protein plasma binding
This was determined as previously described.6

2.5 PK and maximum tolerated dose - mouse
PK studies in female NMRI mice (Figure S1): Test compounds 1, 2 and 10 were dosed SC at 10 mg free base/kg (dose volume 10 mL/kg; dose vehicle: 5% (v/v) DMSO, 40% polyethylene glycol 400 (PEG400) and 55% deionized water) to female NMRI mice (n = 3). Blood samples (10 μL) were taken from each mouse at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8 h post dose (24 h post dose for compounds 2 and 10 only) and mixed with two volumes of deionized water (20 μL). Alternatively, compound 2 was delivered for 48 h via SC implanted ALZET osmotic pumps (10, 25 and 50 mg free base/mL, vehicle: 50% DMSO and 50% deionized water) to the female NMRI mouse (n = 3). Blood samples (10 μL) from each mouse were taken at 1, 3, 6, 24, 30 and 48 h post-dose and mixed with two volumes of deionized water (20 μL).

Maximum tolerated dose
Compound 1 as free base in 5% DMSO; 40% PEG400; 55% MilliQ water was dosed daily subcutaneously with doses of 50 mg/kg, 100 mg/kg and 300 mg/kg for 3 days. No tolerability issues were detected.

2.6 PK and formulation studies - male Sprague rats
Compound 1 was dosed SC at 5 mg free base/kg (dose volume 0.2 or 1 mL/kg; dose vehicle 10% N-methyl-2-pyrrolidinone (NMP), 30% 2-pyrrolidinone, 40% PEG400, 20% deionized water or 10% DMSO, 30% PEG400 and 60% deionized water, respectively) or IM at 5 and 10 mg free base/kg (dose volume 0.2 mL/kg; dose vehicle 10% NMP, 30% 2-pyrrolidinone, 40% PEG400, 20% deionized water or 83.4% 2-pyrrolidinone/polyvinylpyrrolidone (PVP), 16.6% saline or 80% 2-pyrrolidinone/NMP/PVP, 20% (0.05% 2-propanol in saline) to male SD rats (n=3).

Compound 2 was dosed IV at 3 mg free base/kg (dose volume 1 mL/kg; dose vehicle: 10% DMSO, 40% PEG400 and 50% deionized water) or IM at 5, 10 and 20 mg free base/kg (dose volume 0.2
and 0.4 mL/kg; dose vehicle 10% NMP, 30% 2-pyrrolidinone, 40% PEG400, 20% deionized water or 83.4% 2-pyrrolidinone/polyvinylpyrrolidone (PVP), 16.6% saline or 80% 2-pyrrolidinone/NMP/PVP, 20% 0.05% 2-propanol in saline or 40% 0.3M Acetate Buffer, 20% Kolliphor, 20% glycerol formal, 20% 2-pyrrolidinone or 40% 0.3M Acetate Buffer, 20% glycerol formal, 20% 2-pyrrolidinone) to male SD rats (n=3). Blood samples (10 μL) were taken from each rat at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h post dose and mixed with two volumes of deionized water (20 μL).

2.7 Pharmacokinetic studies in cattle (see Table 2). Studies conducted at Ridgeway

Compound 1 was dosed IM at 5 mg free base/kg (dose volume 2mL/10 kg; dose vehicle 10% N-Methyl-2-Pyrrolidinone (NMP), 40% 2-pyrrolidinone, 30% PEG400, 20% deionized water) or IV at 2.5 mg free base/kg (dose volume 0.4 mL/10 kg; 2.5% NMP, 30% PEG400, 37.5% propylene glycol, 30% water by volume) to male ruminating Holstein-Friesian cross animals (n = 4). Blood samples were taken from each animal using 18-G needles into EDTA (K2) vacutainers before dosing and at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96 and 120 h post dose. They were immediately transferred into a cool box containing iced water and were processed within 2 h of collection (Figure S2, Table S1).

Compound 2 was dosed IM at 10 mg free base/kg (dose volume 2 mL/10 kg; dose vehicle 40% 0.3 M Acetate Buffer, 20% Kolliphor EL, 20% glycerol formal, 20% 2-pyrrolidone) or IV at 2 mg free base/kg (dose volume 0.4 mL/10 kg; 40% 0.3 M Acetate Buffer, 20% Kolliphor EL, 20% glycerol formal, 20% 2-pyrrolidone) to male ruminating Holstein-Friesian cross animals (n = 3). Blood samples were taken from each animal using 18-G needles into EDTA (K2) vacutainers before dosing and at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96 and 120 h post dose. They were immediately transferred into a cool box containing iced water and were processed within 2 h of collection.

2.8 Formulation and tolerability studies for compound 2 (NorthWest Biopharm)

A study to identify alternative formulations was conducted. Compound 2, either as the hydrochloride salt or free base, was screened to identify a 50 mg/mL soluble formulation suitable for injection.
Different formulations investigated to reduce injection site tolerability (Table S2)

**Table S2: Salt Formulations Investigated**

| ID | Salt/ Neutral | Formulation |
|----|---------------|-------------|
| 1  | HCl           | Kolliphor EL, glycerol formal, 2-pyrrolidone and 11% water |
| 2  | HCl           | Kolliphor EL, N-methyl pyrrolidone, 2-pyrrolidone, and 11% water |
| 3  | Free base     | Kolliphor EL, N-methyl pyrrolidone, 2-pyrrolidone and 11% water |

The various formulations were then subjected to an injection site tolerability assessment (IST) in cattle to determine if any irritation at the site of injection was evident. Each formulation was administered to two female calves. The calves were cross-breeds (1 x Limousine cross 2 x Hereford cross, 3 x Aberdeen Angus cross). The three formulations of the IVP were supplied formulated ready for administration. Each formulation was administered by single intramuscular injection into the area of the semi-tendinosis muscle of the hind limb and with ascending IM doses of 1 mL, 5 mL and 10 mL. Saline was used as a control. The site of administration for each formulation on each study day was specified in the table below (Table S3).

**Table S3: Sites of Administration for Each Formulation of Compound 2**

| Study Day | T01/Animal 1 (Formulation 1) | T02/Animal 2 (Formulation 2) | T03/Animal 3 (Formulation 3) |
|-----------|------------------------------|------------------------------|------------------------------|
| 0         | 1ml (lower left)             | 1ml (lower left)             | 1ml (lower left)             |
| 3         | 5ml (upper left) saline (upper right) | 5ml (upper left) saline (upper right) | 5ml (upper left) saline (upper right) |
| 7         | N/A                          | 10ml (lower right) saline (lower left) | 10ml (lower right) saline (lower left) |
| Study Day | T01/Animal 5 (Formulation 1) | T02/Animal 6 (Formulation 2) | T03/Animal 7 (Formulation 3) |
| 10        | 1ml Highest non-reactive dose (lower left) | 1ml Highest non-reactive dose (lower left) | 1ml Highest non-reactive dose (lower left) |
| 13        | 5ml Highest non-reactive dose (upper left) Saline (upper right) | 5ml Highest non-reactive dose (upper left) Saline (upper right) | 5ml Highest non-reactive dose (upper left) Saline (upper right) |
| 17        | 10ml Highest non-reactive dose (lower right) Saline (lower left) | 10ml Highest non-reactive dose (lower right) Saline (lower left) | 10ml Highest non-reactive dose (lower right) Saline (lower left) |
On the day of administration, prior to administration of test compounds to any animal and at 30 min and 1 h (± 5 min) and at 3 and 5 h (± 10 min) after administration, a whole blood sample was collected from that animal. This sample was used to determine creatinine kinase (CK) levels, a marker of muscle damage, and levels of compound 2 in the animal at each collection timepoint.

Approximately 5 mL of whole blood was collected from the jugular vein using an appropriate needle and syringe. The sample was divided between two 1.3 mL heparinised blood tubes and one 1.3 mL EDTA (K2) tube. Immediately after collection, the EDTA (K2) tube was placed into iced water. Thereafter, 2 x 0.5 mL of this blood was accurately pipetted into two Eppendorf tubes each containing 1.0 mL of distilled water (also accurately pipetted and stored in a refrigerator). These tubes were pre-labelled for each animal and placed in a rack so that the samples, when frozen, remained at the bottom of the tubes. The blood and water were mixed well by pipetting gently up and down. The samples were then placed in a freezer (-15°C to -20°C). One aliquot of each sample was shipped frozen to the University of Dundee, UK for assessing concentration of compound 2. All cattle blood samples were analysed by UPLC-MS/MS on a Xevo™ TQ-S Mass Spectrometer (MSn) (Waters Corporation, USA). The key Pharmacokinetic parameters were calculated using appropriate software (PK solutions, Summit Research Services, USA).

Following completion of the final clinical assessment of the site of administration (at least 72 h after the last IVP administration to that animal), each animal was euthanised by overdose of barbiturate. Barbiturate was administered via the jugular vein. The site of each IVP administration was dissected from the muscle mass in the form of a block of muscle approximately 2 cm³. Any lesions and their extent at the site of administration were recorded and a digital colour photograph of the lesion was captured and identified with the animal number and the time and date of the photograph. The clinical observations for each formulation are presented below:

- **Formulation 1**: Following a 5 mL injection with formulation 1 (Compound 2 hydrochloride salt in Kolliphor EL, Glycerol Formal, 2-Pyrrolidone and 11% water suitable for injection), an 8 cm swelling appeared at the injection site. Thus, further study of this formulation was stopped prior to proceeding to 10 mL dosing.
• **Formulation 2**: Following a 5 mL injection with formulation 2 (compound 2 hydrochloride salt in Kolliphor EL, N-methyl pyrrolidone, 2-pyrrolidone, and 11% water suitable for injection), a 2-4 cm swelling appeared at the injection site, however, no pain or lameness observed. In contrast, no injection site changes were evident following an injection of 10 mL and there were no signs of associated pain or lameness.

• **Formulation 3**: Administration of a 5 mL injection of formulation 3 (compound 2 free base in Kolliphor EL, N-methyl pyrrolidone, 2-pyrrolidone and 11% water suitable for injection) resulted in a 1-2 cm swelling and a 10 mL injection caused 4 cm swelling.

It should be noted that swellings of <5 cm in these studies are not considered significant. Creatine kinase levels were also markedly lower with formulations 2 and 3 when compared to those in animals treated with formulation 1.

During the study all calves remained clinically well with only local injection site reactions. Blood concentration at each dose volume for each formulation are summarised in Table S3. In parallel, all the formulations were assessed in rats (IM) and the results were comparable to those in cattle, observationally and with regard to creatine kinase levels. These observations offer confidence that rats can be used as a model animal in the drug development path going forward prior to studies in cattle.

**Method (IM)**

**Comparative cattle PK (Northwestern Biopharm)**

The pharmacokinetics for compound 2 when dosed as free base in the best tolerated formulation: Kolliphor EL, N-methyl pyrrolidone, 2-pyrrolidone and 11% water at 5 mg/kg was assessed, IM compared to SC. Mean pharmacokinetic parameters are presented in Table S4. Bioanalysis of blood samples was conducted at the University of Dundee.

**2.9 Salt screen**

A screen of salt forms as alternatives to the hydrochloride was carried out at Drugabilis. Ten pharmaceutically acceptable counter ions were assessed and three were selected for further study
(citrate, fumarate and phosphate) (Figure 9). These alternative salt forms of compound 2 were prepared and formulated in the best vehicle determined by injection site tolerability analysis (0.3M sodium acetate buffer, Kolliphor EL, N-methyl pyrrolidone, 2-pyrrolidone, and 11% water suitable for injection). Rat IM pharmacokinetics was then carried out with each salt to determine the salt form to use for efficacy. None of these salt forms offered any improvement in rat IM pharmacokinetic profile over the free base in the tolerated vehicle (Figure S7).

3. In vivo efficacy studies

The T. congolense (IL3000) and T. vivax (ILRAD V34) cell lines⁷-⁸, both originating from Kenya, were used in the following in vivo mouse studies. For studies in cattle, either Trypanosoma congolense (savannah) KONT 2/133, 224) or T. vivax STIB 719 was used.

3.1 Mouse efficacy studies for T. brucei brucei.

Mouse efficacy studies for T. brucei brucei were carried out as previously detailed.⁹

3.2 NMRI mouse efficacy studies for T. congolense and T. vivax.

Mouse models of infections were carried out using standardized protocols. Briefly, 1 mL samples of cryopreserved T. congolense, or T. vivax-infected blood harvested directly from an infected mouse, were diluted with Hanks Balanced Salt Solution plus 20 mM glucose (HBSS+G) to 5 x 10⁴ trypomastigotes/mL (5 x 10⁵ for T. vivax). Female mice (NMRI) were inoculated IP on day 0 with 0.2 mL/animal (equivalent to ~ 1 x 10⁴ T. congolense or ~ 1 x 10⁵ T. vivax per animal). The level of parasitaemia was inspected microscopically on day 3 using tail blood smears, counting 20 high powered fields (× 400 magnification). For untreated or treated animals whose tail smears exceeded 50 parasites/field, precise parasite levels were established using a Neubauer haemocytometer; mice with a parasite burden over 1 x 10⁸ were promptly and humanely terminated (T. congolense only, from our experience the mice will not survive for a further 24 h). Alternatively, in the case of T. vivax-infected mice the immune system will bring the infection to a sub-lethal level (below limit of detection)
followed by cycles of high and low parasitaemia. Eventually mice infected with *T. vivax* succumb to the infection between 30 – 60 days post-infection.

**Suppression of parasitaemia - mouse**

On day 3 post-infection, animals were randomly allocated to groups, with 3 mice/group. Infected animals were dosed with one of the following: diaminazene diaceturate IP - 40 mg/kg once only as a control (early-stage trypanocide used to clear the parasites in the systemic circulation and in tissues other than CNS), compound 1 (SC) at 3, 10, 30 and 50 mg free base/kg twice and once daily for 4 d or a single dose, or compound 2 (SC) at 3 and 10 mg free base /kg once daily for 4 d, once daily for 2 d or a single dose, one group of 3 mice acted as an untreated control group. The dose solutions were prepared daily, using 5% DMSO: 40% PEG400: 55% MilliQ H₂O for test compounds and 10% DMSO: 90% peanut oil for standard drugs. Alternatively, compound 2 was delivered for 48 h via SC implanted ALZET osmotic pumps (6, 12, 25 and 50 mg free base/mL, vehicle: 50% DMSO and 50% deionized water). Mean blood concentration data presented in Figure S8.

Response to treatment was determined by monitoring parasitaemia twice a week until day 21. After day 21, the mice were checked once a week until 63 d post-treatment. Mice surviving to the end of the experiment, and with blood smears clear of parasites, were considered cured.

**3.3 Cattle efficacy for compound 1 (Clinvet)**

In each of the cattle efficacy studies (3.3, 3.4 and 3.5), efficacy was assessed primarily on absence of recrudescence of parasitaemia for 100 days after treatment administration together with improvements in Packed Cell Volume, body weight and clinical signs of infected compared with uninfected animals. Assessment of parasitaemia was based on phase contrast buffy coat examinations and confirmation by PCR at 100 d.

Friesian-Holstein calves, weaned for at least 2 months and with a minimum age of 4 months, housed in a fly-proof building, were infected with *Trypanosoma congolense* (*savannah*) KONT 2/133, at a dose of 110,000 parasites (study day 0) via IV infusion. The parasites were obtained from a donor calf. This isolate is resistant to diminazene (20 mg/kg) and isometamidium (1 mg/kg) in cattle, and diminazene (7 mg/kg) and isometamidium (>1 mg/kg) in mice. Calves were parasitaemic by study day 6. Calves (*n* = 5) were treated with compound 1 (5 mg/kg, SC, twice 12 h apart) on study
day at 25 mg/ml in NMP/2-pyrrolidinone/PEG400 water 10/40/30/20. Control groups were comprised of un-infected calves, infected but un-treated calves (n = 6) and calves treated with diminazene (n = 3) or isometamidium (n = 3). The calves were randomised to treatments. Calves were monitored daily for parasitaemia and packed cell volume (PCV) via blood sampling and through clinical symptoms.

One day after treatment, 80% of the animals in the treatment group were negative for parasites. After 3 d, 100% of the animals in the treatment groups were negative for parasites. After 5 d, 40% of the animals were positive for parasites. After 6 d, 80% were positive and after 8 days, 100% were positive.

3.4 Cattle efficacy for compound 2, intravenous infusion (Clinvet)

Suppression of parasitaemia - cattle

Friesian-Holstein calves (similar age and housing to those in 3.3) were infected on day 0. Animals were infected with fresh cattle blood from animals infected with T. congolense KON 2/133 (n = 4) or T. vivax STIB 719 (n = 4). Compound 2 was administered by IV bolus followed by a 24 h IV infusion, followed by another IV bolus and then an additional 12 h intravenous infusion. The concentration of the infusion was 2 mg of free base/ml). The total dose was 14.8 mg/kg. Compound 2 was administered in 5% (w/v) glucose. Control cattle (n = 3 for T. congolense and n = 3 for T. vivax) were dosed with saline. Blood samples were collected for PK analysis from treated animals before administration of the compound and at the following times post-administration of the first bolus (+1, +2, +4, +8, +10, +12, +16, +20, +24 (before second bolus), +25, +26, +28, +32, +34, +36, +40, +48 h). Cattle were monitored for packed cell volume (PCV) daily from days 1 to 14 and then 3 times weekly. They were monitored for parasitaemia 3 times per week, from day 1 to day 114.

3.5 Cattle efficacy for compound 2, intramuscular (CIRDES)

Felani-zebu cattle were housed in a fly-proof facility, acclimatised for 15 d prior to the study, cattle were treated with an anthelmintic, an ectoparasiticide and an antibiotic. Donor animals were infected with stabilates (frozen stocks) of either T. congolense KOT256 (resistant to 7 mg/kg diminazene in cattle) or T. vivax Gando Bongaly (resistant to diminazene 7 mg/kg and to isometamidium 1 mg/kg
in cattle). Blood from the donor animals, diluted with phosphate buffered glucose was used to infect recipient animals IV via the jugular vein. Infection was with approximately 100,000 fresh viable parasites. The compounds were given IM. Each injection was up to 10 mL and given in neck in the following order: left anterior third, right anterior third, left posterior third and right posterior third. Animals over 240 kg required a fifth injection site for the IVP, and this was given in the left or right hind leg. Cattle were randomised to treatments. Parasitaemia was monitored 3 times per calendar week, though collection of blood and assessment of clinical symptoms. Blood samples were also taken for PK analysis (before treatment, 1, 2, 4, 8, 12, 16, 20, 24, 32, 36, 48, 56 and 60 h post treatment). Cattle were also monitored for PCV, clinical symptoms, body weight and temperature (Tables S5 and S6).

4. Mode of action studies

4.1 Resistant line generation

Compound-resistant cell lines were generated by sub-culturing a clone of T. brucei in the continuous presence of 2. Starting at sub-lethal concentrations, drug concentrations in 3 independent cultures were increased in a step-wise manner, usually by 2-fold. When parasites were able to survive and grow in concentrations of drug equivalent to more than 10 times the established EC50 value, the resulting cell lines were cloned by limiting dilution in the presence of compound. Three clones (RES I-III) were selected for further biological study.

4.2 Whole genome sequencing and analysis

A standard alkaline lysis protocol was used to isolate genomic DNA from compound-resistant bloodstream T. brucei parasites (~5 x 10⁸). Whole genome sequencing was performed using a HiSeq4000 next generation sequencing platform (Beijing Genomics Institute, Hong Kong). Sequencing reads (100 bp) were aligned to the Trypanosoma brucei TREU927 genome (v39; tritrypDB) using Bowtie2¹⁰ and Samtools¹¹ software. Samtools and BCFtools¹² were used to call single nucleotide polymorphisms (SNP) and indels compared with the wildtype starter clone, where
overall quality score (QUAL) was >100. Artemis was used to analyse chromosome and gene copy number variation (CNV), as well as visualisation of SNPs.

4.3 RIT-seq screening, Illumina sequencing and analysis

The RIT-seq library screening was performed as described previously. The RNAi library was maintained with blasticidin (1 µg ml$^{-1}$) and phleomycin (1 µg ml$^{-1}$) at a minimum of 2 x 10$^7$ cells. Following tetracycline (1 µg ml$^{-1}$) induction for 24 h, compound 2 (4 nM), and compound 12 (98 nM), were added to cultures and supplemented with fresh compound and tetracycline as required. DNA was extracted from compound-selected cells and RNAi target fragments were amplified from compound-selected parasites by PCR using the Lib2f and Lib2r primers. PCR products were fragmented and sequenced with an Illumina HiSeq platform at BGI (Beijing Genomics Institute). Reads were mapped to the T. brucei 927 reference genome (v39; TriTrypDB [http://tritrypdb.org/]) using Bowtie 2 software, with the following parameter: --very-sensitive-local. Following manipulation with SAMtools the alignment files were searched with a custom script to identify reads with the following barcode: GCCTCGCGA. The total and barcoded reads were then quantified using the Artemis genome browser.

T. brucei CRK12 (Tb927.11.12310) editing

For TbCRK12 mutagenesis at positions L$^{482}$ or G$^{492}$, we used a bloodstream-form T. brucei Cas9 inducible expression system. The gRNA construct was assembled by annealing Fwgcrk12_b (AGGGAATGTGCTCATTAATAGGGA) and Rvgcrk12_b (AAACCTCCCTATTATGAGCACATT) and the resulting gRNA fragment was ligating to the BbsI-digested pTT$^{sgRNA}$ construct. Correct integration was confirmed by sequencing selected clones, which were then transfected into 2T1$^{TT-Cas9}$ cells. The repair templates were CRK12_482temp:

(CCGTGATGTCAAGGCTGACATTGNNATCAAACAGAGACGGCAGCGTCCAGCTTGGGAGCAC TTCG)

and

CRK12_492temp:

(AGTGCAAGGCTGACATTGCTCATCAAACAGAGACGGCAGCGTCCAGCTTGGGAGCAC TTTGTCGACCTTTTGGGAGC)
with randomised codons at the appropriate positions shown in bold; synonymous edits that should promote insertion of randomised codons are underlined. Each template (40 µg) was delivered to the sgRNA strain by electroporation 24 h after Cas9-induction, using tetracycline at 1 µg/mL; tetracycline was removed at this point. Compound 2 selection (10 nM) was applied 6 h later. After 5 days of selection, drug-resistant cultures were subcloned and six clones were selected from each experiment. DNA was extracted from each subclone and a specific portion of the TbCRK12 gene encompassing the edited region was PCR-amplified and sequenced.

4.4 SILAC pulldown

*Trypanosoma brucei* procyclic form parasites were grown in SDM-79 media (SILAC SDM-79, 2 g/L NaHCO₃, 7.5 mg haemin, 1% glutamax and 15% dialysed FBS) with either light or heavy isotopes for 6-9 divisions (6 days) to a density of ~3 x 10⁷ cells/ml. A total of 3 x 10⁸ cells were washed thrice with PBS. Buffer 1 (0.1 mM tosyl phenylalanyl chloromethyl ketone, 1 µg/ml leupeptin, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1x Roche Complete protease inhibitors) was added to the parasite pellet, followed by Buffer 2 (100 mM Tris pH 7.5, 10% glycerol, 300 mM NaCl, 3 mM MgCl₂, 1.6% IGEPAL® CA-630, 2 mM dithiothreitol) and incubated at 4°C for 20 min. Lysates were centrifuged at 100,000 g at 4°C for 1 h. Beads (blank and compound-bound) were washed twice with water and twice with lysis buffer (Buffer 1 and 2). Lysates were incubated with 2 mg blank beads for 30 min at 4°C. Lysates were then incubated with either 10 µM DDD00770675 or 1% DMSO for 30 min at 4°C. Finally, lysates were incubated with 2 mg of bound compound for 1 h at 4°C. Beads were washed three times with wash buffer (0.5% IGEPAL® CA-630, 50 mM Tris pH 8.0, 5 mM EDTA, 1 mg/ml BSA) and twice with Tris-buffered saline. Samples were combined, run 1.5 cm into a Brs-TRIS 10% acrylamide gel and stained with Coomassie quick reagent for 1 h. Gel bands were subjected to in-gel reduction with 10 mM dithiothreitol, alkylation with 50 mM iodoacacetamide and digestion with 12.5 µg/mL trypsin (Pierce) for >16h at 37°C. Recovered tryptic peptides were vacuum dried.
4.5 Peptide fractionation and LC-MS/MS analysis

Peptides were fractionated using High pH RP Chromatography. A C18 Column from Waters (XBridge peptide BEH, 130Å, 3.5 µm 2.1 x 150 mm, Waters, Ireland) with a guard column (XBridge, C18, 3.5 µm, 2.1 x10 mm, Waters) were used on a Ultimate 3000 HPLC (Thermo-Scientific). Buffers A and B used for fractionation consist, respectively, of (A) 10mM ammonium formate in milliQ water pH 9.5 and (B) 10 mM ammonium formate, pH 9.5 in 90% acetonitrile. Fractions were collected using a WPS-3000FC auto-sampler (Thermo-Scientific) at 1 min intervals. Column and guard column were equilibrated with 2% Buffer B for twenty minutes at a constant flow rate of 0.2 mL/min. Peptides (190 µL) were injected onto the column, and the separation gradient was started 1 min after sample was loaded onto the column. Peptides were eluted from the column with a gradient of 2% Buffer B to 60% Buffer B in in 53 min. The column was washed for 15 min in 100% Buffer B and equilibrated at 2% Buffer B for 20 min. The fraction collection started 1 min after injection and stopped after 70 min (total 70 fractions, 200 µl each). The total number of fractions concatenated was set to 10 and the content of the fractions was dried and suspended in 25 µL of 1% formic acid prior to analysis with LC-MS. Analysis of peptide readout was performed on a Q Exactive™ plus, Mass Spectrometer (Thermo Scientific) coupled with a Dionex Ultimate 3000 RS (Thermo Scientific). LC buffers used are the following: Buffer A (0.1% formic acid in Milli-Q water (v/v)) and Buffer B (83.7% acetonitrile and 0.1% formic acid in Milli-Q water (v/v)). Aliquots of 15 µl per fraction were loaded at 10 µL min⁻¹ onto a trap column (100 µm x 2 cm, PepMap nanoViper C18 column, 5 µm, 100 Å, Thermo Scientific) which was equilibrated with 98% Buffer A. The trap column was washed for 5 min at the same flow rate and then the trap column was switched in-line with a Thermo Scientific, resolving C18 column (75 µm x 50 cm, PepMap RSLC C18 column, 2 µm, 100 Å). The peptides were eluted from the column at a constant flow rate of 300 nL/min with a linear gradient from 2% buffer to 35% Buffer B in 125 min, and then to 98% Buffer B by 127 min. The column was then washed with 98% Buffer B for 20 min and re-equilibrated in 2% Buffer B for 17 min. Q-Exactive plus was used in data dependent mode. A scan cycle comprised MS1 scan (m/z range from 335-1600, with a maximum ion injection time of 20 ms, a resolution of 70,000 and automatic gain control (AGC) value of 1×106) followed by 15 sequential dependent MS2 scans (with an isolation window set to 1.4 Da, resolution at 17500, maximum ion injection time at 100 ms and AGC 2×105. To ensure mass accuracy, the mass
spectrometer was calibrated on the first day that the runs were performed. LC-MS analysis was performed by the FingerPrints Proteomics Facility (University of Dundee).

4.6 Data analysis

MS data analysis was performed using the software MaxQuant (http://maxquant.org/, version 1.6.2.6a). Carbamidomethyl (C), oxidation (M), acetyl (Protein N-term), deamidation (NQ) and Gln-> pyro-Glu were set as a variable modification. Proteins were identified by searching a protein sequence database containing *T. brucei brucei* TREU927 annotated proteins (downloaded from TriTrypDB 42, http://www.tritrypdb.org). Trypsin/P was selected as the digestive enzyme with two potential missed cleavages. The FDR threshold for peptides and proteins was 0.01. FTMS MS/MS mass tolerance was set to 10 ppm and ITMS MS/MS mass tolerance was 0.6 Da. Normalized SILAC ratios were calculated using unique and razor peptides and required a minimum of two SILAC pairs. Data was visualized using Perseus 1.6.2.1 (https://maxquant.org/perseus/).

5. Molecular modelling

The sequence of *Tb*CRK12 kinase domain (residues 323-685 of the Tb927.11.12310 entry in the TriTrypDB database) was used to identify homologues performing a Blast search on the Protein Data Bank proteins. The structure of the *human* CRK13 structure complexed with ADP (PDB ID 5EFQ), displaying a sequence identity of 35%, was selected as template for homology modelling. The sequence alignment between the Tb (target) and human (template) sequences was generate using the multisequence viewer tool in the Schrödinger modelling platform (Schrödinger 301 Release 2019-3: Schrödinger, LLC, New York, NY, 2020) and was manually curated to ensure that the highly conserved kinase catalytic residues were correctly aligned (Figure S10). The homology model of the *Tb*CRK12:ADP structure was generated using the energy-based method implemented in the Schrödinger protein structure prediction module Prime. Low energy conformers for compound 2 were generated using LigPrep (Schrödinger 301 Release 2019-3: Schrödinger, LLC, New York, NY, 2020). The prepared ligand was then docked into the ATP binding of the *Tb*CRK12 model using Glide (Schrödinger 301 Release 2019-3: Schrödinger, LLC, New York, NY, 2020) in standard
precision mode. No distance or hydrogen-bond constraints were applied. The 10 best scoring docking poses obtained using the default settings were further optimised by post-docking molecular energy minimisation. A 20 Å cubic box centred on the centroid of the ADP molecule was used to generate the docking grid. Within the grid both hydroxyl and thiol groups were allowed to rotate.

6. Supplementary figures

Figure S1: The pharmacokinetics of compound 1 following single SC administration to the female NMRI mouse at 10 mg free base/kg (n=3).

Figure S2: The pharmacokinetics of compound 1 over 24 h following IV (left panel) and IM (right panel) administration to male Fresian-Holstein cattle (n=4) at 2.5 mg free base/kg. For IV bolus injection, the vehicle was 2.5% NMP, 30% PEG400, 37.5% Propylene Glycol, 30% deionised water. For IM injection, the dose vehicle was 10% N-Methyl-2-Pyrrolidinone (NMP), 40% 2-pyrrolidinone, 30% PEG400, 20% deionized water
A: 83.4% PVP/2-pyrrolidone; 16.6% saline
B: 80% vehicle 1 (50% N-methyl-2-pyrrolidone: 50% 2-pyrrolidone); 20% vehicle 2 (5% 2-propanol: 95% 0.86% sodium chloride solution).

**Figure S3:** The pharmacokinetics of compound 1 following single IM administration to the female Sprague Dawley rat at 5 mg free base/kg using two different formulations (n=3 per formulation). Free concentration in blood <EC₉₀ at 3 or 4 h, respectively.

**Figure S4:** Comparison of the pharmacokinetics of compound 1 following single IM administration to female Sprague Dawley rats or to male Fresian-Holstein cattle following IM administration. Both experiments were conducted using the same formulation (10% NMP, 30% 2-pyrrolidinone, 40% PEG400, 20% MilliQ H₂O) at the same dose and dose level (5 mg/kg; 25 mg/mL; 0.2 mL/kg). Comparable mean drug exposure was observed (Rat: Cmax 92ng/mL, AUC₀₋₈ 21265ng-min/mL; Cattle: Cmax 133ng/mL, AUC₀₋₈ 20924ng-min/mL).
Figure S5: (A) The pharmacokinetics of compound 2 following single IM administration to female Sprague Dawley rats at 10 mg free base/kg using three different formulations (n=3 rats per formulation). Shown in black: 40% 0.3 M Acetate Buffer/ 20% Kolliphor EL/ 20% Glycerol Formal/ 20% 2-pyrrolidone. Shown in white: 50% 0.3 M Acetate Buffer/ 25% Glycerol Formal/ 25% 2-pyrrolidone. Shown in grey: 83.4% 2-pyrrolidinone/polyvinylpyrrolidone/ 16.6% saline. (B) The blood concentration time profile of compound 2 in female NMRI mice following single SC administration at 10 mg free base/kg using used 5% DMSO/ 40% PEG400/ 55% MilliQ water. AUC$_{0-24}$ 129608 ng-min/mL; Cmax 818 ng/mL; Cav 78 ng/mL; Cmin (8 h) 10 ng/mL.

Figure S6: Blood concentration time profile of compound 2 in Friesian cattle following single IM administrations at 10 mg free base/kg. AUC 439785 ng-min/mL; Cmax 1723 ng/mL; Cav 253 ng/mL; Cmin (24 h) 20 ng/mL.
**Figure S7**: Exposures of different salt formulations when dosed to male Sprague Dawley rats IM. Prototype formulation was 0.3 M Acetate Buffer (40%), Kolliphor EL (20%), Glycerol Formal (20%) and 2-Pyrrolidone (20%). Free base and salt forms were prepared and formulated in the best vehicle from the injection site tolerability work (Kolliphor EL, N-methyl Pyrrolidone, 2-Pyrrolidone, and 11% water suitable for injection).
Figure S8: Compound 2 mean (n=3) blood concentration time profile following dosing in T. congolense infected mice via an ALZET pump implant. Using a 2001D ALZET pump (nominal pumping rate of 8μl/hr): 2mg/ml white; 6 mg/mL black; 12 mg/mL grey. Using a 1003D ALZET pump (nominal pumping rate of 1μL/hr): 25 mg/mL blue; 50 mg/mL red. Formulation used was 50:50 DMSO/sterile water. All mice at each dose level were cured.
Figure S9: Blood concentration time profiles for compound 2 in (a) *T. congolense-* and (b) *T. vivax*-infected cattle (CIRDES, Burkina Faso). Kolliphor EL, *N*-methyl pyrrolidone, 2-pyrrolidone and 11% water as the formulation using the free base.

Figure S10. Sequence alignment between the target *TbCRK12* (Tb927.11.12310 entry in the TriTrypDB database) and the template *hCDK13* (from PDB entry 5EFQ) used for the generation of the homology model.
7. LCMS chromatograms for compounds used in in vivo studies

Figure S11. LCMS chromatogram for compound 1.
Figure S12. LCMS chromatogram for compound 2.
Figure S13. LCMS chromatogram for compound 3.
Figure S14. LCMS chromatogram for compound 4.
Figure S15. LCMS chromatogram for compound 5.
Figure S16. LCMS chromatogram for compound 8.
Figure S17. LCMS chromatogram for compound 10.
8. Supplementary tables

**Table S4**: Pharmacokinetic parameters of compound 1 following IV (A) or IM (B) administration in male Fresian-Holstein cattle (n=4) at 2.5 or 5 mg free base/kg, respectively.

A: IV

| Parameters        | Animal no. |        |        |        |        |
|-------------------|------------|--------|--------|--------|--------|
|                   | 2754 | 7462 | 1753 | 6752   |
| Clnb (mL/min/kg)  | 24   | 20   | 21   | 15     |
| Vdss (L/kg)       | 3.5  | 1.8  | 2.5  | 1.7    |
| Half-life (h)     | 2.4  | 1.2  | 1.8  | 1.7    |
| AUC$_{0-\infty}$ (ng-min/mL) | 100,000 | 120,000 | 120,000 | 160,000 |

B: IM

| Parameters        | Animal no. |        |        |        |        |
|-------------------|------------|--------|--------|--------|--------|
|                   | 2754 | 7462 | 1753 | 6752   |
| Cmax (ng/mL)      | 70   | 128  | 118  | 216    |
| Tmax (h)          | 0.5  | 0.5  | 0.5  | 0.25   |
| AUC$_{0-\infty}$ (ng-min/mL) | 21,000 | 26,000 | 15,000 | 22,000 |
| F (%)             | 10   | 11   | 7    | 7      |
**Table S5:** Pharmacokinetic parameters of compound 2 following IV (A) or IM (B) administration at 2 or 10 mg free base/kg to the male Friesian-Holstein cattle (n=3), respectively.

**A:**

| Parameters       | Animal no. | Mean (n=3) |
|------------------|------------|------------|
|                  | 1644       | 3646       | 7643       |
| C1b (mL/min/kg)  | 14         | 21         | 11         | 15         |
| Vdss (L/kg)      | 2          | 3          | 2          | 3          |
| Half-life (h)    | 6          | 6          | 3          | 4          |
| AUC 0-∞ (ng-min/mL) | 140,000   | 94,000     | 180,000    | 130,000    |

**B:**

| Parameters       | Animal no. | Mean (n=3) |
|------------------|------------|------------|
|                  | 1644       | 3646       | 7643       |
| Cmax (ng/mL)     | 950        | 1,700      | 2,600      | 1,700      |
| Tmax (h)         | 2          | 1          | 1          | 1          |
| AUC 0-∞ (ng-min/mL) | 530,000   | 510,000    | 460,000    | 490,000    |
| F (%)            | 78         | 75         | 66         | 74         |
**Table S6**: Pharmacokinetic data for compound 2 when administered to cattle IM.

| IM injection (mL) | Time (min) | Formulation 1 | Formulation 2 | Formulation 3 |
|-------------------|------------|----------------|----------------|----------------|
|                   |            | HCl salt       | HCl salt       | Free Base     |
|                   |            | Kolliphor, Glycerol Formal, 2-Pyrollidone, 11% Water | Kolliphor, NMP, 2-Pyrollidone, 11% Water | Kolliphor, NMP, 2-Pyrollidone, 11% Water |
| pre-dose          | ≤ 3        | ≤ 3            | ≤ 3            |
| 30                | 12         | 32             | 107            |
| 60                | 13         | 25             | 4              |
| 180               | 42         | 42             | 3              |
| 300               | 20         | 32             | ≤ 3            |
| 30                | 20         | 12             | 8              |
| 60                | 15         | 13             | 4              |
| 180               | 36         | 26             | 8              |
| 300               | 20         | 17             | 11             |
| pre-dose          | ≤ 3        | ≤ 3            | ≤ 3            |
| 30                | 54         | 27             | 111            |

| IM injection (mL) | Time (min) | Scaled concentration (ng/mL) |
|-------------------|------------|------------------------------|
|                   | Calf 0242  | Calf 0091                    | Calf 0481 |
| pre-dose          | ≤ 3        | ≤ 3                          | ≤ 3       |
| 30                | 12         | 32                           | 107       |
| 60                | 13         | 25                           | 4         |
| 180               | 42         | 42                           | 3         |
| 300               | 20         | 32                           | ≤ 3       |
| pre-dose          | ≤ 3        | ≤ 3                          | ≤ 3       |
| 30                | 119        | 93                           | 93        |
| 60                | 68         | 257                          | 50        |
| 180               | 50         | 181                          | 46        |
| 300               | 55         | 121                          | 38        |
| pre-dose          | ≤ 3        | ≤ 3                          | ≤ 3       |
| 30                | 119        | 93                           | 93        |
| 60                | 94         | 68                           | 68        |
| 180               | 90         | 48                           | 48        |
| 300               | 109        | 29                           | 29        |

| IM injection (mL) | Time (min) | Scaled concentration (ng/mL) |
|-------------------|------------|------------------------------|
|                   | Calf 0110  | Calf 0090                    | Calf 0131 |
| pre-dose          | ≤ 3        | ≤ 3                          | 4         |
| 30                | 20         | 12                           | 8         |
| 60                | 15         | 13                           | 6         |
| 180               | 36         | 26                           | 8         |
| 300               | 20         | 17                           | 11        |
| pre-dose          | ≤ 3        | ≤ 3                          | ≤ 3       |
| 30                | 54         | 27                           | 111       |
**Table S7**: Pharmacokinetic Parameters for Compound 2 when dosed to Aberdeen Angus crossbreed and Limousin crossbreed cattle at 5 mg/kg as the free base using Kolliphor EL, N-methyl Pyrrolidone, 2-Pyrrolidone and 11% water as the vehicle. Dosing was IM or SC.

|   | IM  | SC  | IM  | SC  |
|---|-----|-----|-----|-----|
| 5 | 60  | 60  | 47  | 81  |
|   | 180 | 74  | 74  | 64  |
|   | 300 | 83  | 84  | 44  |
| 10 | pre-dose | - | ≤ 3 | 4 |
|    | 30  | -   | 29  | 96  |
|    | 60  | -   | 67  | 64  |
|    | 180 | -   | 107 | 75  |
|    | 300 | -   | 157 | 131 |

|   | AUC, ng-min/mL | Cmax (obs) ng/mL | Tmax (h) |
|---|----------------|------------------|----------|
| IM | 190,000        | 360              | 0.5      |
| SC | 58,000         | 86               | 3.0      |
Table S8: Concentration of compound 2 (ng/mL) in blood following dosing in *T. congolense* infected cattle (CIRDES, Burkina Faso).

| Animal | T1 | T2 |
|--------|----|----|
|        | Compound 2 (single dose IM) | Compound 2 (dosed twice IM) |
| Time (h) | | |
| 0 | <LOQ | <LOQ | 10 | <LOQ | <LOQ | 5 | 39 | 4 | <LOQ | <LOQ |
| 1 | 413 | 439 | 593 | 312 | 351 | 868 | 2224 | 914 | 251 | 275 |
| 2 | 525 | 523 | 511 | 436 | 555 | 967 | 644 | 1156 | 234 | 381 |
| 4 | 473 | 518 | 379 | 453 | 391 | 920 | 383 | 827 | 279 | 312 |
| 8 | 159 | 260 | 178 | 174 | 252 | 349 | 169 | 295 | 154 | 204 |
| 12 | 71 | 108 | 98 | 104 | 138 | 167 | 90 | 162 | 75 | 141 |
| 16 | 47 | 67 | 64 | 48 | 101 | 96 | 281 | 916 | 312 | 415 |
| 20 | 22 | 39 | 40 | 28 | 39 | 58 | 261 | 703 | 293 | 278 |
| 24 | 11 | 30 | 46 | 23 | 34 | 62 | 132 | 279 | 146 | 170 |
| 32 | 8 | 15 | 33 | 12 | 33 | 24 | 54 | 122 | 53 | 63 |
| 48 | 18 | 5 | 14 | 5 | 6 | 9 | 7 | 14 | 15 | 12 |
| 56 | 3 | 21 | 4 | 3 | 4 | 6 | 8 | 20 | 15 | 19 |
| Efficacy | no | no | no | no | no | no | no | yes | no | no |

| Animal | T3 | T4 |
|--------|----|----|
|        | Dosed twice with saline | Dosed twice with saline, followed by two doses of compound 2 rescue IM 5 days later |
| Time (h) | | |
| 0 | <LOQ | 5 | 4 | 15 | <LOQ | <LOQ | 5 | 15 | 5 | 11 |
| 1 | 4 | <LOQ | 47 | 434 | 16 | 930 | 3 | 912 | 3 | 870 |
| 2 | 14 | <LOQ | 15 | 781 | <LOQ | 1440 | <LOQ | 1176 | <LOQ | 1011 |
| 4 | 6 | <LOQ | 344 | 4 | 1120 | <LOQ | 2680 | <LOQ | 695 |
| 8 | <LOQ | <LOQ | 4 | 155 | <LOQ | 339 | 3 | 373 | <LOQ | 275 |
| 12 | 4 | <LOQ | 5 | 60 | 6 | 137 | <LOQ | 205 | <LOQ | 116 |
| 16 | <LOQ | 11 | 4 | 45 | 14 | 1041 | 19 | 1287 | 7 | 1062 |

S-38
| Time | Value | Value | Value | Value | Value | Value | Value | Value | Value | Value | Value | Value | Value | Value | Value | Value | Efficacy |
|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|---------|
| 20   | 357   | <LOQ  | 6     | <LOQ  | 19    | 4     | 494   | 4     | 484   | <LOQ  | 441   | <LOQ  | <LOQ  | <LOQ  | 258   | 72     | 3      |
| 24   | <LOQ  | 72    | 3     | 14    | 13    | 276   | 12    | 236   | <LOQ  | 441   | 4     | <LOQ  | <LOQ  | 258   | 72     | 3      |
| 32   | <LOQ  | <LOQ  | 3     | 39    | 22    | 105   | 51    | 76    | 71    | 54    | 5     | 5     | 3     | 104   | 18     | <LOQ   |
| 48   | 3     | 5     | 4     | 8     | 104   | 18    | <LOQ  | 16    | 28    | 11    | 2     | 3     | 18    | <LOQ  | 16     | 236    |
| 56   | <LOQ  | 4     | <LOQ  | 3     | 728   | 5     | 12    | <LOQ  | 16    | 28    | 11    | <LOQ  | <LOQ  | 258   | 72     | 3      |

Efficacy: no no no no yes no yes no no no no

LOQ = 2 ng/ml
**Table S9**: Concentration of compound 2 (ng/mL) in blood following dosing in *T. vivax* infected cattle (CIRDES, Burkina Faso).

| Animal | T5 Compound 2 dosed once IM | T6 Compound 2 dosed twice IM |
|--------|-----------------------------|-------------------------------|
| Time (h) | 1907 | 1915 | 1918 | 1932 | 1933 | 1940 | 1902 | 1916 | 1926 | 1929 |
| 0 | <LOQ | ND | ND | <LOQ | 5 | 29 | <LOQ | <LOQ | 3 |
| 1 | 514 | 2485 | 437 | 379 | 907 | 418 | 880 | 704 | 583 |
| 2 | 702 | 2304 | 504 | NS | 1370 | 656 | 1081 | 1183 | 782 |
| 4 | 815 | 1505 | 657 | 702 | 1394 | 643 | 806 | 1208 | 726 |
| 8 | 485 | 960 | 342 | 357 | 703 | 373 | 475 | 854 | 280 |
| 12 | 313 | 623 | 186 | 235 | 291 | 235 | 196 | 520 | 143 |
| 16 | 211 | 567 | 133 | 180 | 166 | 110 | 748 | 703 | 240 |
| 20 | 153 | 457 | 77 | 67 | 136 | 69 | 797 | 999 | 368 |
| 24 | 137 | 353 | 169 | 53 | 623 | 546 | 479 | 1055 | 279 |
| 32 | 70 | 291 | 31 | 136 | 26 | 21 | 187 | 414 | 97 |
| 48 | 25 | NS | 32 | 22 | 26 | 5 | 92 | 130 | 26 |
| 56 | 20 | NS | 32 | 12 | 9 | 5 | 48 | 131 | 15 |

**Efficacy**

| Animal | T5 | T6 |
|--------|----|----|
| Efficacy | No | ND |

ND, not determined
| Animal | T7       | T8       | T9       |
|--------|----------|----------|----------|
|        | Dosed twice with saline | Dosed twice with saline, followed by single rescue dose of compound 2 IM 4 days later | Dosed twice with saline, followed by single rescue dose of compound 2 IM 4 days later |
|        |          |          |          |
|        |          |          |          |

| Time (h) | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 | T7 | T8 | T9 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 0        | <LOQ| <LOQ| <LOQ| 11  | 3   | 28  | <LOQ| <LOQ| <LOQ| 59  | 1   | <LOQ| <LOQ| 1125 | <LOQ| 883 | <LOQ| 493 | 3   | 462 | 2   | 5   | 4   | <LOQ| 1994| <LOQ| 1265| <LOQ| 905 | <LOQ| 875 | 4   | 6   | 7   | 7   | 1056| <LOQ| 876 | <LOQ| 688 | 3   | 735 | 8   | 14  | <LOQ| 465 | <LOQ| 417 | <LOQ| 391 | <LOQ| 415 | 12  | <LOQ| 4   | 173 | <LOQ| 167 | 3   | 193 | <LOQ| 144 | 16  | 19  | 7   | <LOQ| 107 | 14  | 635 | <LOQ| 613 | 14  | 427 | 20  | 4   | <LOQ| 85  | <LOQ| 854 | 3   | 624 | <LOQ| 416 | 24  | 38  | 5   | 30  | 63  | 101 | 395 | 120 | 305 | 14  | 208 | 32  | 3   | 201 | <LOQ| 30  | 1165| 161 | 8   | 104 | 3   | 86  | 48  | 21  | 54  | 39  | 10  | 6   | 82  | 114 | 68  | 24  | 36  | 56  | 2   | 27  | 31  | 5   | 4   | 22  | <LOQ| 13  | 3   | 17  | Efficacy | No  | no  | No  | ND  | No  | ND  | No  | No  | No  | no  |

LOQ = 2 ng/ml
**Table S10:** Metabolism of compound 2 to compound 8.

| Time (h) | Compound 8 Peak area | Compound 2 Peak area | % cpd 8/ cpd 2 by peak area | Compound 8 peak area | Compound 2 peak area | % cpd 8/ cpd 2 by peak area |
|----------|----------------------|----------------------|-----------------------------|----------------------|----------------------|-----------------------------|
| 0        | 0                    | 0                    | 0                           | 285                  | 0                    | 0                           |
| 1        | 4,000                | 180,000              | 2.2                         | 6,900                | 160,000              | 4.2                         |
| 2        | 6,600                | 240,000              | 2.8                         | 11,000               | 230,000              | 4.8                         |
| 4        | 9,000                | 210,000              | 4.3                         | 19,000               | 260,000              | 7.2                         |
| 8        | 2,000                | 69,000               | 2.9                         | 19,000               | 160,000              | 12                          |
| 12       | 1,500                | 32,000               | 4.7                         | 15,000               | 100,000              | 15                          |
| 16       | 1,000                | 21,000               | 5.0                         | 11,000               | 68,000               | 17                          |
| 20       | 790                  | 9,900                | 8.0                         | 8,300                | 49,000               | 17                          |
| 24       | 380                  | 5,100                | 7.5                         | 6,300                | 44,000               | 14                          |
| 32       | 270                  | 3,600                | 7.5                         | 4,100                | 23,000               | 18                          |
| 48       | 130                  | 8,000                | 1.6                         | 1,600                | 8,300                | 19                          |
| 56       | 130                  | 1,600                | 8.2                         | 660                  | 6,600                | 10                          |
Table S11: Identification of *T. brucei* proteins that bind specifically to resin-bound 13. Data derived from analysis of chemo proteomic data from stable isotope labelled *T. brucei* procyclic lysate incubated with either compound 2 or DMSO followed by incubation with compound-resin (compound 13). Resin-bound proteins were analysed by LS-MS/MS with a high DMSO/compound ratio indicating specific binding to 13. Data shown for Log$_2$ DMSO/compound >1.5, 2.8-fold enrichment. Proteins shown were identified by the detection of at least 2 unique peptides and 2 ratio H/L counts. Data is the mean of three experiments.

| Gene ID         | Log$_2$ ratio | Protein description                  | Abbreviation | Unique peptides |
|-----------------|---------------|--------------------------------------|--------------|-----------------|
| Tb927.1014800   | 4.15          | MAP kinase 9, putative               | MAPK9        | 10              |
| Tb927.6.3110    | 3.57          | Cdc-2 related kinase 11             | CRK11        | 7               |
| Tb927.11.12310  | 3.47          | Cdc2-related kinase 12              | CRK12        | 19              |
| Tb927.11.14030  | 3.16          | Cyclin 9                            | CYC9         | 6               |
| Tb927.10.16030  | 2.84          | MAP kinase 4                        | MAPK4        | 5               |
| Tb927.1.700     | 1.92          | Phosphoglycerate kinase             | PGK          | 4               |
| Tb927.7.4400    | 1.85          | Inositol polyphosphate kinase-like protein | IPPK      | 7               |
| Tb927.10.5140   | 1.62          | MAP kinase 6                        | MAPK6        | 30              |

Table S12: Summary of read counts and genome coverage following whole genome sequencing.

| Cell line | Number of reads | Read length | Percentage mapped | Fold coverage | Loss of SNP heterozygosity | Gain of SNP heterozygosity | Gain of SNP homozygosity |
|-----------|-----------------|-------------|-------------------|---------------|---------------------------|---------------------------|-------------------------|
| WT        | 40710212        | 100         | 80.89             | 90            | -                         | -                         | -                       |
| RES1      | 40380322        | 100         | 81.42             | 90            | 852                       | 3                         | 0                       |
| RES2      | 41040418        | 100         | 81.47             | 92            | 26                        | 3                         | 0                       |
| RES3      | 41109768        | 100         | 81.11             | 91            | 856                       | 2                         | 1                       |
Table S13: Summary of mutations found in compound 2-resistant lines.

| Chromosome | Nucleotide position | Gene ID       | WT | Mut | Amino acid change | Resistant lines | Gene name     |
|------------|---------------------|---------------|----|-----|-------------------|----------------|---------------|
|            |                     |               |    |     |                   | R1 | R2 | R3 |               |               |
| Homozygous mutations (coding regions only) |
| 11         | 3291310             | Tb927.11.12310 | G  | C   | Gly492Ala         | -  | -  | 1/1 | CRK12         |
| Heterozygous mutations (coding regions only) |
| 4          | 1272440             | Tb927.4.4590  | A  | G   | Leu474Pro         | 0/1| 0/1| 0/1| AGC family Ser/Thr kinase |
| 11         | 2749965             | Tb927.11.10300| G  | T   | Glu56STOP         | 0/1| 0/0| 0/1| Hypothetical   |
| 11         | 3291279             | Tb927.11.12310| C  | T   | Leu482Phe         | 0/0| 0/1| 0/0| CRK12         |
| 11         | 3291310             | Tb927.11.12310| G  | C   | Gly492Ala         | 0/1| 0/1| -  | CRK12         |
Table S14: Extended hit list for Compound 12 RIT-seq analysis (>1000 RPKM 'barcoded reads' cut-off)

| Gene ID          | Gene description                                             | All mapped reads | Barcoded reads only | Fragments |
|------------------|--------------------------------------------------------------|------------------|---------------------|-----------|
| Tb927.9.13470    | hypothetical protein, conserved 99232 948093 198910 296661 3 |                  |                     |           |
| Tb927.10.16125   | hypothetical protein 17238 7381 52829 3196 0                |                  |                     |           |
| Tb927.10.11720   | CW type-Zinc finger, putative 18666 178339 52130 78400 1 |                  |                     |           |
| Tb927.1.4250     | hypothetical protein 21077 203698 36461 54701 3             |                  |                     |           |
| Tb927.10.16120   | inosine-5'-monophosphate dehydrogenase 13916 87335 26099 24976 8 |                  |                     |           |
| Tb927.11.11530   | histone acetyltransferase, putative 11758 87022 18400 19861 3 |                  |                     |           |
| Tb927.11.14870   | NAD+ synthase, putative 10691 38581 9004 5460 1             |                  |                     |           |
| Tb927.6.1070     | hypothetical protein 3250 34355 7981 13377 4                 |                  |                     |           |
| Tb927.4.990      | hypothetical protein 2787 11967 7709 4677 0                 |                  |                     |           |
| Tb927.10.16120   | inosine-5'-monophosphate dehydrogenase 13916 87335 26099 24976 8 |                  |                     |           |
| Tb927.11.11530   | histone acetyltransferase, putative 11758 87022 18400 19861 3 |                  |                     |           |
| Tb927.11.14870   | NAD+ synthase, putative 10691 38581 9004 5460 1             |                  |                     |           |
| Tb927.6.1070     | hypothetical protein 3250 34355 7981 13377 4                 |                  |                     |           |
| Tb927.4.990      | hypothetical protein 2787 11967 7709 4677 0                 |                  |                     |           |
| Tb927.10.16120   | inosine-5'-monophosphate dehydrogenase 13916 87335 26099 24976 8 |                  |                     |           |
| Tb927.11.11530   | histone acetyltransferase, putative 11758 87022 18400 19861 3 |                  |                     |           |
| Tb927.11.14870   | NAD+ synthase, putative 10691 38581 9004 5460 1             |                  |                     |           |
| Tb927.6.1070     | hypothetical protein 3250 34355 7981 13377 4                 |                  |                     |           |
| Tb927.4.990      | hypothetical protein 2787 11967 7709 4677 0                 |                  |                     |           |
| Tb927.10.16120   | inosine-5'-monophosphate dehydrogenase 13916 87335 26099 24976 8 |                  |                     |           |
| Tb927.11.11530   | histone acetyltransferase, putative 11758 87022 18400 19861 3 |                  |                     |           |
| Tb927.11.14870   | NAD+ synthase, putative 10691 38581 9004 5460 1             |                  |                     |           |
| Tb927.6.1070     | hypothetical protein 3250 34355 7981 13377 4                 |                  |                     |           |
| Tb927.4.990      | hypothetical protein 2787 11967 7709 4677 0                 |                  |                     |           |
| Tb927.10.16120   | inosine-5'-monophosphate dehydrogenase 13916 87335 26099 24976 8 |                  |                     |           |
| Tb927.11.11530   | histone acetyltransferase, putative 11758 87022 18400 19861 3 |                  |                     |           |
| Tb927.11.14870   | NAD+ synthase, putative 10691 38581 9004 5460 1             |                  |                     |           |
Table S15: Extended hit list for Compound 2 RIT-seq analysis (>1000 RPKM 'barcoded reads' cut-off)

| Gene ID       | Gene description                                      | All mapped reads | Barcoded reads only |
|---------------|-------------------------------------------------------|------------------|---------------------|
|               | RPKM        | Actual | RPKM  | Actual | Fragments |
| Tb927.9.13470 | hypothetical protein, conserved                      | 30403            | 305080             | 94211   | 163003    | 7       |
| Tb927.10.6600 | hypothetical protein, conserved                      | 40913            | 183455            | 73811   | 60990     | 1       |
| Tb927.11.14870| NAD+ synthase, putative                              | 36458            | 138184            | 34763   | 23909     | 2       |
| Tb927.11.11530| histone acetyltransferase, putative                 | 14460            | 112397            | 34314   | 43909     | 3       |
| Tb927.1.4250  | hypothetical protein, conserved                      | 10010            | 101604            | 20465   | 33845     | 6       |
| Tb927.10.8100 | hypothetical protein, conserved                      | 7555             | 26247             | 15417   | 11585     | 1       |
| Tb927.10.8060 | hypothetical protein, conserved                      | 5755             | 26247             | 15417   | 11585     | 1       |
| Tb927.10.13710| Activator of Hsp90 ATPase, N-terminal/Activator of Hsp90 ATPase homolog 1-like protein | 981                | 4183                | 2256              | 1584     | 5       |
| Tb927.7.2760  | Mitochondrial ribosomal protein L22                 | 1001              | 3587                | 2169              | 1431     | 0       |
| Tb927.10.2240 | RNA binding protein                                 | 830              | 6281                | 1922              | 2546     | 4       |
| Tb927.2.4340  | hypothetical protein, conserved                      | 464              | 2572                | 1850              | 1730     | 1       |
| Tb927.9.13030 | QA-SNARE protein putative                           | 725              | 2851                | 1656              | 1069     | 1       |
| Tb927.10.4590 | mitochondrial inner membrane signal peptidase, putative | 390              | 1041                | 1257              | 613      | 2       |
| Tb927.2.2015  | hypothetical protein                                | 386              | 124                 | 1169              | 69       | 2       |
| Tb927.11.2860 | hypothetical protein, conserved                      | 1335             | 13207               | 1125              | 1672     | 1       |
| Tb927.10.11720| CW-type Zinc Finger, putative                       | 413              | 4145                | 1103              | 1873     | 3       |
| Tb927.10.9400 | splicing factor 1                                   | 533              | 1861                | 1034              | 591      | 1       |

**Primary 'hit'>1 fragment per gene**

**Secondary 'hit' ≤ 1 fragment per gene**

**Adjacent hit**
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