Cyclin-dependent kinase 12 is a drug target for visceral leishmaniasis

Susan Wyllie1, Michael Thomas1, Stephen Patterson1, Sabrina Crouch2, Manu De Rycker1, Rhiannon Lowe3, Stephanie Gresham3, Michael D. Urbaniak1,4, Thomas D. Otto5,9, Laste Stojanovski1, Frederick R. C. Simeons1, Sujatha Manthri1, Lorna M. MacLean1, Fabio Zuccotto1, Nadine Homeyer1, Hannah Pflaumer4, Markus Boesche4, Lalitha Sastry1, Paul Connolly7, Sebastian Albrecht1, Matt Berriman3, Gerard Drewes6, David W. Gray1, Sonja Ghidelli-Disse5, Susan Dixon8, Jose M. Fiandor2, Paul G. Wyatt1, Michael A. J. Ferguson1, Alan H. Fairlamb1, Timothy J. Miles2, Matt Berriman5, Gerard Drewes6, David W. Gray1, Sonja Ghidelli-Disse5, Susan Dixon8, Jose M. Fiandor2, Paul G. Wyatt1, Michael A. J. Ferguson1, Alan H. Fairlamb1, Timothy J. Miles2, Matt Berriman5, Gerard Drewes6, David W. Gray1, Sonja Ghidelli-Disse5, Susan Dixon8, Jose M. Fiandor2, Paul G. Wyatt1, Michael A. J. Ferguson1, Alan H. Fairlamb1, Timothy J. Miles2

Visceral leishmaniasis causes considerable mortality and morbidity in many parts of the world. There is an urgent need for the development of new, effective treatments for this disease. Here we describe the development of an anti-leishmanial drug–like chemical series based on a pyrazolopyrimidine scaffold. The leading compound from this series (7, DDD853651/GSK3186899) is efficacious in a mouse model of visceral leishmaniasis, has suitable physicochemical, pharmacokinetic and toxicological properties for further development, and has been declared a preclinical candidate. Detailed mode–of–action studies indicate that compounds from this series act principally by inhibiting the parasite cdc–2–related kinase 12 (CRK12), thus defining a druggable target for visceral leishmaniasis.

Leishmania parasites cause a wide spectrum of human infections ranging from the life-threatening visceral disease to disfiguring mucosal and cutaneous forms. Leishmania spp. are obligate intracellular parasites of the vertebrate reticuloendothelial system, where they multiply as amastigotes within macrophage phagolysosomes; transmission is by blood-sucking sandflies, in which they proliferate as extracellular promastigotes.

Visceral leishmaniasis, resulting from infection with Leishmania donovani and L. infantum, causes 20,000–40,000 deaths annually according to the WHO (World Health Organization; http://www.who.int/leishmaniasis/en/), of which approximately 60% occur in India, Bangladesh and Nepal1. In 95% of cases, death can be prevented by timely and appropriate drug therapy2. However, current treatment options are far from ideal, with outcomes depending on several factors including geographical location, the immune status and other co-morbidities of the patient, and the disease classification. None of the current front-line treatments for visceral leishmaniasis—amphotericin B (liposomal or deoxycholate formulations), miltefosine, paromomycin and antimonials—is ideal for use in resource-poor settings, owing to issues such as teratogenicity, cost, resistance and/or clinical relapse, prolonged treatment regimens and parental administration3,5,7. Thus, there is an urgent need for new treatment options for visceral leishmaniasis, particularly oral drugs. Unfortunately, there are currently, to our knowledge, no new therapeutics in clinical development and only a few in preclinical development. There is a paucity of well-validated molecular drug targets in Leishmania, and the molecular targets of the current clinical molecules are unknown. Recent studies8 identified the proteasome as a promising therapeutic target for the treatment of visceral leishmaniasis as well as other kinetoplastid infections, and this currently represents the most robustly validated drug target that has been reported in these parasites. Furthermore, whole-cell (phenotypic) screening programs have been hindered by extremely low hit rates7.

Previously, we reported the identification of a diaminothiazole series from a compound screen against Trypanosoma brucei GSK3 kinase (TbGSK3)8. During compound optimization, it became clear that the anti-trypanosomal activity of this series was driven, at least in part, by off-target activity. The early compounds showed activity against T. brucei bloodstream trypanosomes in viability assays, but showed little activity against L. donovani axenic amastigotes (for example, compound 1). Modification of the core structure, while retaining the functions of the hydrogen bond donor and acceptor, gave a bicyclic compound series (Fig. 1), one of which (compound 2) showed very weak activity against L. donovani axenic amastigotes, but was inactive against the more clinically relevant intra-macrophage amastigotes.Appending a sulfonamide to the cyclohexyl ring resulted in compound 3, which was active against L. donovani amastigotes in both the axenic and intra-macrophage assays9,10 and selectively active against L. donovani compared to the THP-1 mammalian host cells used in the assay. Replacement of the iso-butyl substituent on the pyrazole ring with an aromatic substituent and the benzyl group on the sulfonamide with a trifluoropropyl substituent resulted in compound 4, which had marginally more activity. Notably, this compound demonstrated more than 70% parasite reduction in a mouse model of visceral leishmaniasis when dosed orally, providing proof of concept in an animal model for this series. Replacing the pyridyl group with a 2-methoxyphenyl and the trifluoropropyl group with an iso-butyl group gave our most potent compound 5, which had a half-maximum effective concentration (EC₅₀) value of 0.014 μM in the intra-macrophage assay. Compound 5 was metabolically unstable, although it demonstrated more than 95% parasite reduction when dosed in a hepatic cytochrome P450

There are amendments to this paper

https://doi.org/10.1038/s41586-018-0356-z

© 2018 Springer Nature Limited. All rights reserved.
reSeArcH ON reSeArcH

1.2–1.5 μM in the cidal assay with an EC50 value of 1.4 μM. In the cidal assay, a higher cell density and improved detection limit is used compared to the axenic assay, allowing distinction between cytostatic and cytocidal compounds. HBA, hydrogen bond acceptor; HBD, hydrogen bond donor.

Fig. 1 | The evolution of the pyrazolopyrimidine series to give the development compound. Potencies against axenic amastigotes, intra-macrophage amastigotes and against THP-1 cells are shown; data show the geometric mean of at least three independent replicates. In the cidal assay, a higher cell density and improved detection limit is used compared to the axenic assay, allowing distinction between cytostatic and cytoxic compounds. HBA, hydrogen bond acceptor; HBD, hydrogen bond donor.

© 2018 Springer Nature Limited. All rights reserved.
a

| Experiment no. | Dose (mg kg⁻¹) | Frequency of treatment | Treatment duration (days) | Reduction in parasite load (%) | LDU control animals |
|---------------|---------------|------------------------|---------------------------|-------------------------------|--------------------|
| 1             | 50            | BID                    | 5                         | 98                            | 370,000            |
| 2             | 25            | BID                    | 10                        | 99                            | 610,000            |
| 3             | 10            | BID                    | 10                        | 49                            | 500,000            |
| 4             | 3             | BID                    | 4                         | 4                             | 500,000            |
| 5             | 25            | UID                    | 5                         | 50                            | 570,000            |
| 6             | 25            | UID                    | 10                        | 89                            | 630,000            |
| 7             | 50            | UID                    | 10                        | 95                            | 370,000            |

b

Fig. 2 | Efficacy of compound 7 in a mouse model of visceral leishmaniasis. Each arm was carried out with five mice. a, Reduction in parasite load for various dose regimens. BID, twice daily dosing; UID, once daily dosing. LDU, Leishman–Donovan units (the number of amastigotes per 500 nucleated cells multiplied by the organ weight in grammes). b, Dose–response curve for twice daily dosing for 10 days. Error bars show the s.d. for n = 5 mice. c, Given dose required to give a particular reduction in parasite load for twice daily dosing for 10 days. The reported ED₅₀ value for miltefosine in a mouse model is 27 mg kg⁻¹ once a day.

This was the series developed from a known protein kinase scaffold, Kinobead technology was used to determine whether compound 7 inhibits human protein kinases. These experiments indicated that compound 7 interacted with four human kinases—MAPK11, NLK, MAPK4 and CDK7—at concentrations within multiples of the predicted clinical dose (Supplementary Table 1). However, the extent of inhibition of these human kinases is not sufficient to preclude clinical development of the molecule, and no marked inhibition of other human kinases was detected in the Kinobead assays. Non-GLP preclinical assessment of cardiovascular effects and genotoxicity did not reveal any issues that would prevent further development. In addition, there were no notable adverse effects in a rat seven-day repeat-dose oral toxicity study with respect to clinical chemistry and histopathology at all doses tested. Both the in vivo efficacy and safety profile of compound 7 support progression to definitive safety studies.

Mode of action studies

Determining the mode of action of new chemical series can greatly benefit drug discovery campaigns. Because there is no blueprint to establish the mode of action of bioactive small molecules, several complementary methods were used. Representative pyrazolopyrimidine analogues (4, 5, 6 and 7) from the drug discovery program were used as chemical tools (Fig. 1), as was compound 8, in which the diaminocyclohexyl group was replaced by an aminopiperidine amide. These compounds showed very good activity correlation between the intra-macrophage, axenic amastigote and promastigote assays, giving us confidence to use the extracellular parasite forms (promastigote) for mode-of-action studies where it was not possible to use the intracellular forms (amastigote) (Supplementary Table 2).

As a first step towards identifying the target(s) of the leishmanicidal pyrazolopyrimidine series, structure–activity relationships were used to inform the design of analogues containing a polyethylene glycol (PEG) linker (9, 11 and 12; Extended Data Fig. 2), which were then covalently attached to magnetic beads to allow for chemical proteomics. First, beads derivatized with compound 9 were used to pull-down proteins from SILAC (stable isotope labelling by amino acids in cell culture)-labelled L. donovani promastigotes labelled in the presence (‘light-labelled lysate’) or absence (‘heavy-labelled lysate’) of 10 μM compound 10, a structurally related, bioactive derivative of compound 9. After combining the bead eluates and performing proteomic analyses, proteins that bound specifically to the pyrazolopyrimidine pharmacophore could be distinguished from proteins that bound non-specifically to the beads by virtue of high-light tryptic peptide isotope ratios. These experiments identified CRK12, CRK6, CYC9, CRK3, MPK9, CYC6 and a putative STE11-like protein kinase (LinJ.24.1500) as specific binders to the compound 9-derivatized beads (log of heavy-light ratio > 2.8; 7-fold enrichment) (Supplementary Fig. 5 and Supplementary Table 3).

Second, pull-down experiments were conducted with beads derivatized with compounds 9, 11 or 12, followed by competition studies with compounds 5, 8 or 10, respectively. Adherent proteins were washed off the beads, digested with trypsin and labelled with isobaric tandem mass tags. Comparison of the labelled peptides derived from experiments with and without competition, by liquid chromatography–tandem mass spectrometry identified proteins that are likely to bind specifically to the immobilized ligands. Potential candidates identified included CRK3, CRK6, CRK12, CYC3, CYC6, CYC9, MPK9 and MPK5 and several hypothetical proteins (Supplementary Fig. 6 and Supplementary Table 4). We also investigated immobilizing the compound at an alternative position on the scaffold and this gave a similar binding profile (Supplementary Fig. 6 and Supplementary Table 4), further validating the approach. These results are consistent with previous studies that report that the pyrazolopyrimidine core binds to protein kinases.

The presence of cdc2-related kinases (CRK3, CRK6 and CRK12) and cyclins (CYC3, CYC6 and CYC9) in the initial target list led us to analyse the effects of pyrazolopyrimidines (5, 7, 8 and 10) on cell-cycle progression in L. donovani. Treatment resulted in an accumulation of cells in the G1 and in G2/M phases of the cell cycle, and a decrease in the proportion of cells in S phase (Fig. 3a for compound 7 and Supplementary Fig. 9 for compounds 5, 6 and 8), suggesting arrests in the cell-cycle at G1/S and G2/M phases, consistent with a mode of action via CRK and/or CYC components.

Resistance was generated in L. donovani promastigotes against compounds 4 and 5. A single cloned parental cell line was divided into three individual cultures for each compound, and resistance was generated by exposing parasites to step-wise increasing the concentrations of the compounds. After resistance generation, each independently generated cell line was cloned and three individual clones for each compound (six in total) were selected for in-depth study. The resulting clones demonstrated more than 500-fold and 9–17-fold resistance to compounds 4 and 5, respectively (Extended Data Table 5). Resistance to both compounds was found to be stable over 50 days in culture in the absence of drug pressure and, notably, all clones showed cross-resistance to compounds 4 and 5, and 20–50-fold cross-resistance to compound 7. These data suggest our pyrazolopyrimidines share common mechanisms of resistance and most likely modes of action. Importantly, intracellular amastigotes, derived from the resistant promastigotes, were 8.5-fold and 5-fold resistant to compounds 4 and 5, respectively, compared to wild-type parasites (Extended Data Table 6), strongly suggesting that their mechanism(s) of action are the same in promastigote and intracellular amastigote stages of the parasite.

To gain further insight into the mechanism of action and potential target(s) of this pyrazolopyrimidine series, our six drug-resistant clones underwent whole-genome sequence analysis. A range of mutations, relative to parental clones, were found across the genome (Supplementary Table 5), including a long region with loss of heterozygosity on
numbers are common in *Leishmania* and extra copies of chromosome 9, containing the CRK12 gene, were found in four out of six of the drug-resistant clones (Supplementary Table 7). In addition, three of these four clones had extra copies of chromosome 32, containing the gene for CYC9. Previous studies in *T. brucei* have established that the partner cyclin of CRK12 is CYC9. This suggests that CYC9 may be the cognate cyclin partner for *L. donovani* CRK12.

**Target validation**

To dissect the role of CRK12 and CYC9 in the mechanism of action and resistance of pyrazolopyrimidines, a series of protein overexpression studies were undertaken in *L. donovani* promastigotes. In all cases, overexpression of putative targets was confirmed by increased levels of transcripts in our transgenic cell lines relative to the wild-type cells, as determined by quantitative PCR (qPCR) (Supplementary Table 8).

Counterintuitively, overexpression of wild-type CRK12 rendered the parasites approximately 3-fold more sensitive to compound 5 (Fig. 3b). The overexpression of CYC9 alone had no effect on compound resistance, but co-overexpression of CYC9 and wild-type CRK12 rendered the transgenic parasites around 3-fold more resistant to compounds 5 and 7 (Fig. 3c and Supplementary Table 8). Next, we looked at the mutated (Gly572 to Asp) version of CRK12 (CRK12(G572D)) identified in all of our drug-resistant clones. Overexpression of CRK12(G572D) rendered the parasites around 3.4-fold resistant to compound 5 (Fig. 3d and Supplementary Table 8) and to the preclinical lead compound 7 (Supplementary Table 8), while being equally sensitive to the unrelated nitroimidazole drug fexinidazole sulfone (Supplementary Table 9). Co-overexpression of CRK12(G572D) and CYC9 rendered the parasites 6-fold more resistant to compound 7 and 8-fold more resistant to compound 5. This shift in sensitivity is considerably greater than the 3.4-fold increased resistance observed with parasites overexpressing CRK12(G572D) alone (Fig. 3d). Replacing a single copy of the CRK12 gene with a drug-selectable marker left parasites approximately 2-fold more susceptible to compound 5 than the wild-type cells (Fig. 3e and Supplementary Fig. 10). We were unable to directly replace both endogenous copies of the CRK12 gene, except in the presence of an ectopic copy of the gene, suggesting that the CRK12 gene is essential for the growth and survival of *L. donovani* (Supplementary Fig. 10).

Initially, CRK3 and CRK6 were identified as credible targets based on our collective proteomics datasets, as well as their established roles in kinetoplastid cell cycle regulation. However, whole-genome sequencing, qPCR (Supplementary Fig. 8) and Southern blot (Supplementary Fig. 7) analysis of resistant clones confirmed that mutations within, or amplification of, the CRK3 and CRK6 genes were not responsible for resistance to pyrazolopyrimidines. Direct modulation of CRK3 and CRK6 levels within *L. donovani* promastigotes, by overexpressing overexpression and single-gene knockout parasites, did not alter drug sensitivity (Supplementary Table 8). Overexpression of CRK3 and CRK6 in combination with their cognate cyclin partners CYC6 and CYC3 was not possible because co-overexpression proved toxic. Collectively, these data suggest that the primary mechanism of action of this compound series is unlikely to be via CRK3 or CRK6 inhibition.

Commonly, overexpression of the molecular target of a compound is accompanied by an increase in drug resistance. With this in mind, our collective data strongly suggest that the principal target of our pyrazolopyrimidine series is the CYC9-activated form of CRK12, such that overexpression of CRK12 and CYC9 together provides resistance. This hypothesis is also consistent with the amplification of both CRK12 and CYC9 in resistant parasites; as well as the identification of both proteins in our SILAC and Kinobead proteomic datasets. The fact that overexpression of CYC9 alone has no effect suggests that CYC9 is, to some extent, in excess over CRK12 and thus overexpression of CRK12(G572D) can provide (3-fold) resistance that is increased when additional CYC9 is co-expressed (8-fold). The 'hyper-sensitivity' of parasites overexpressing wild-type CRK12 alone to these compounds remains perplexing. One potential explanation is that wild-type CRK12 bound to a
pyrazolopyrimidine in the absence of a CYC9 subunit is particularly toxic to the parasite. Alternatively, increased levels of CRK12 may well sequester other cyclins, thereby preventing their essential interactions with other CRKs. Further studies will be required to test these hypotheses.

Given that the compounds from this chemical series interacted with protein kinases, in particular CRK12, we used Kinobead technology\(^\text{14,21,22}\) with axenic amastigote extracts to identify pyrazolopyrimidine–binders in the *Leishmania* kinome. These experiments were performed in the presence or absence of an excess of the soluble parent compound 5. All proteins captured by the beads were quantified by tandem mass tag (TMT) labelling of tryptic peptides followed by liquid chromatography–tandem mass spectrometry analysis\(^\text{15}\). CRK12, MPK9, CRK6 and CYC3 (Fig. 4a) were identified, consistent with the other experiments above. A dose–response experiment was performed in which compound 5 was added over a range of concentrations to establish a competition-binding curve and determine a half-maximal inhibition concentration (IC<sub>50</sub>) value (Fig. 4b). The IC<sub>50</sub> values obtained in these experiments represent a measure of target affinity, but are also affected by the affinity of the target for the bead-immobilized ligands. The latter effect can be deduced by determining the depletion of the target proteins by the beads, such that apparent dissociation constant (K<sub>d</sub>) values can be determined that are largely independent from the bead ligand\(^\text{23}\). The apparent K<sub>d</sub> values were 1.4 nM for CRK12, 45 nM for MPK9, 58 nM for CYC3 and 97 nM for CRK6. These values are determined in physiological conditions (substrates, cyclins and ATP) and provide further compelling evidence that the principal target of this compound series is CRK12. Further pull-downs with a resin-bound pyrazolopyrimidine analogue (11) were conducted in parallel with the Kinobead experiments and returned broadly similar results (Fig. 4c, d).

Collectively, our data provide strong evidence that CRK12 forms an important interaction with CYC9: (1) our studies indicate that over-expression of CYC9 together with CRK12 markedly increases resistance to our pyrazolopyrimidine compounds; (2) in several of our compound-resistant cell lines, additional copies of chromosome 32, containing the CYC9 gene, were found; (3) in the related organism *T. brucei*, CYC9 was confirmed as the partner cyclin for CRK12; and (4) in several chemical proteomics studies, CYC9 was identified as binding to immobilized compounds from our pyrazolopyrimidines alongside CRK12.

**Modelling**

A homology model was built for *L. donovani* CRK12 using the structure of human cyclin dependent kinase 9 (CDK9, Protein Data Bank (PDB) code 4BCF) as a template. (Notably, compound 7 showed an IC<sub>50</sub> value of greater than 20 μM against CDK9 in the Kinobead assay.) A combination of docking studies, molecular dynamics simulation and free-energy calculations indicated that the most likely binding mode is that shown in Fig. 5 (see Supplementary Information for discussion). With very few exceptions, the binding modes of protein kinase inhibitors are highly conserved across kinase family members; searching the protein database revealed a related 5-amino-pyrazolopyrimidine, which bound to ALK in a very similar fashion (PDB code 4Z5S, ligand 4LO). In our proposed binding mode, the bicyclic scaffold interacts with the hinge residues establishing two hydrogen bonds between the sp<sup>2</sup> pyrimidine nitrogen in position 6 and the backbone NH of Ala566, and between the pyrazole NH in position 1 and the backbone carbonyl oxygen of Ala564 (Fig. 5b). A third hydrogen bond is also established between the amino NH in position 5 and the backbone carbonyl oxygen of Ala566. The substituent in position 3 of the pyrazole ring is directed towards the ATP back-pocket interacting with the gatekeeper residue (Phe563). This binding mode is consistent with the analogues 9, 11 and 12 retaining binding affinity, with the PEG linkers being attached to water-accessible parts of the core. The Gly572Asp mutation that causes resistance to the pyrazolopyrimidine series is located at the end of the hinge region, nine residues from the gatekeeper. In the Gly572Asp mutant, the negatively charged side chain of the aspartic acid is positioned in close contact to the oxygen atoms of the sulfonamide moiety, leading to an unfavourable electrostatic interaction.

**Discussion**

New oral drugs for visceral leishmaniasis, particularly those capable of treating ongoing outbreaks in East Africa, are urgently needed. Effective drugs will make a notable difference to treatment outcomes for this devastating parasitic disease. With the ultimate goal of elimination of visceral leishmaniasis, several treatment options will be required. We have identified a pyrazolopyrimidine series that shows the potential to treat visceral leishmaniasis. Our studies indicate that the principal mechanism of action of our pyrazolopyrimidine compounds is by the inhibition of CRK12, defining CRK12 as one of very few chemically
validated drug targets in *Leishmania*. Furthermore, our data indicate that CYC9 is the definitive partner cyclin for CRK12. The physiological function(s) of CRK12 and CYC9 have yet to be determined and the availability of our inhibitory pyrazolopyrimidines should assist in probing this aspect of parasite biology.

It is clear from our collective chemical proteomics studies that the pyrazolopyrimidines also interact with other *Leishmania* protein kinases, in particular CRK6 and CRK3, albeit with considerably lower affinities than for CRK12. Although CRK12 is undoubtedly the principal target of this compound series, we cannot rule out the possibility that underlying this mechanism of action is an element of polypharmacology. Indeed, the inhibition of secondary kinase targets may be responsible for some of the phenotypic effects observed in drug-treated parasites, such as cell cycle arrest.

Compound 7 is being advanced towards human clinical trials and is currently undergoing preclinical development. The data generated so far provide a reason to believe that compound 7 has the potential to fulfill the community target product profile (see https://www.dndi.org/diseases-projects/leishmaniasis/tpp-vl). However, as a systematic approach to drug discovery is relatively new in this neglected disease, there are outstanding questions that can only be answered as the compound progresses through development.

**Reporting summary**

Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

Compound 7 is currently in preclinical development and full disclosure of the synthesis of this compound has been included in this publication. All reasonable requests for the other key tool molecules disclosed in this manuscript will be met subject to an appropriate material transfer agreement in place between all parties.

**Online content**

Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at https://doi.org/10.1038/s41586-018-0356-z.

Received: 9 August 2017; Accepted: 11 June 2018; Published online 25 July 2018.

1. Alvar, J. et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS ONE* 7, e385671 (2012).
2. Ritmeijer, K. & Davidson, R. N. Royal Society of Tropical Medicine and Hygiene joint meeting with Médicins Sans Frontières at Manson House, London, 20 March 2003: field research in humanitarian medical programmes. Médicins Sans Frontières interventions against kala-azar in the Sudan, 1989–2003. *Trans. R. Soc. Trop. Med. Hyg.* 97, 609–613 (2003).
3. Sundar, S. et al. Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clin. Infect. Dis.* 55, 543–550 (2012).
4. den Boer, M. L., Alvar, J., Davidson, R. N., Ritmeijer, K. & Balasegaram, M. Developments in the treatment of visceral leishmaniasis. *Expert Opin. Emerg. Drugs* 14, 395–409 (2009).
5. Mueller, M. et al. Unresponsiveness to AmBisome in some Sudanese patients with kala-azar. *Trop. Plann. Sci. Soc. Trop. Med. Hyg.* 101, 19–24 (2007).
6. Khare, S. et al. Proteasome inhibition for treatment of leishmaniasis, Chagas disease and sleeping sickness. *Nature* 537, 229–233 (2016).
7. Don, R. & Isot, J.-R. Screening strategies to identify new chemical diversity for drug development to treat kinetoplastid infections. *Parasitol. Today* 21, 140–146 (2014).
8. Woodland, A. et al. From on-target to off-target activity: identification and optimisation of *Trypanosoma brucei* GSK3 inhibitors and their characterisation as anti- *Trypanosoma brucei* drug discovery lead molecules. *ChemMedChem* 8, 1127–1137 (2013).
9. De Ryck, M. et al. Comparison of a high-throughput high-content intracellular *Leishmania donovani* assay with an axenic amastigote assay. *Anticancer. Agents Chemother.* 57, 2913–2922 (2013).
10. Nuiss, A. et al. Development and validation of a novel *Leishmania donovani* screening cascade for high-throughput screening using a novel axenic assay with high predictivity of leishmanial intracellular activity. *PLoS Negl. Trop. Dis.* 9, e0004955 (2015).
11. Henderson, C. J., Pess, G. J. & Wolf, C. R. The hepatic cytochrome P450 reductase null mouse as a tool to identify a successful candidate entity. *Toxicol. Lett.* 162, 111–117 (2006).
12. Miles, T. J. & Thomas, M. G. Pyrazolo[3,4-d]pyrimidin derivative and its use for the treatment of leishmaniasis. *WIPO patent* 2005/121107 (2005).
13. Dong, G., Jiang, N. & Roberts, J. L. Pyrazolo pyrimidines. *WIPO patent* 2006/074984 (2006).
14. Rogers, M. B. et al. Chromosome and gene copy number variation allow major structural changes between species and strains of *Leishmania*. *Genome Res.* 21, 2129–2142 (2011).
15. Downie, T. et al. Whole genome sequencing of multiple *Leishmania donovani* clinical isolates provides insights into population structure and mechanisms of drug resistance. *Genome Res.* 21, 2143–2156 (2011).
16. Monneret, S. et al. Identification and functional characterisation of CRK12:CYC9, a novel cyclin-dependent kinase (CDK)-cyclin complex in *Trypanosoma brucei*. *PLoS ONE* 8, e67327 (2013).
17. Hassain, F., Fergusson, D., Grant, K. M. & Mottram, J. C. The CRK2 protein kinase is essential for cell cycle progression of *Leishmania mexicana*. *Mol. Biochem. Parasitol.* 113, 189–198 (2001).
18. Tu, X. & Wang, C. C. Pairwise knockdowns of cdc2-related kinases (CRKs) in *Trypanosoma brucei* identified the CRKs for G1/S and G2/M transitions and demonstrated distinctive cytokinetic regulations between two developmental stages of the organism. *Eukaryot. Cell* 4, 755–764 (2005).
19. Ménard, G. et al. Optimized chemical proteomics assay for kinase inhibitor screening. *J. Proteome Res.* 14, 1574–1586 (2015).
20. Bargamin, G. et al. A selective inhibitor irrelevant of PI3Kα dependence of Tε17 cell differentiation. *Nat. Chem. Biol.* 5, 576–582 (2012).
21. Bantschett, M. et al. Chemoproteomics profiling of HDAC inhibitors reveals selective targeting of HDAC complexes. *Nat. Biotechnol.* 29, 285–295 (2011).
22. Bradley, D. J. & Kirkley, J. Regulation of *Leishmania* populations within the host. I. The variable course of *Leishmania donovani* infections in mice. *Clin. Exp. Immunol.* 30, 119–129 (1977).
23. Croft, S. L., Snowdon, D. & Yardley, Y. The activities of four anticancer alkylsulphophospholipids against *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma brucei*. *J. Antimicrob. Chemother.* 38, 1041–1047 (1996).
24. Seifert, K. & Croft, S. L. In vitro and in vivo interactions between miltefosine and other antileishmanial drugs. *Antimicrob. Agents Chemother.* 50, 1–9 (2006).
25. Escobar, P., Yardley, Y. & Croft, S. L. Activities of hexadecylphosphocholine (miltefosine), AmBisome, and sodium stibogluconate (Pentostam) against *Leishmania donovani* in immunodeficient scid mice. *Antimicrob. Agents Chemother.* 45, 1872–1875 (2001).

**Acknowledgements**

The authors acknowledge the Wellcome Trust for funding (grants 092340, 105021, 100476, 101842, 079838 and 098051).

**Reviewing information**

Nature thanks R. Guy, J. Mottram and the other anonymous reviewer(s) for their contribution to the peer review of this work.

**Author contributions**

In brief, S.W., M.D.U., T.O.D., H.P., M.B. and S.M. carried out the mode of action, genomic and proteome studies. M.T., S.P. and S.A. carried out the chemistry studies. M.D.R., S.M., L.M.M. and L.S. carried out the parasite screening. S.C., L.S., F.R.C.S. and P.C. carried out the drug metabolism and pharmacokinetics studies and N.H. carried out the molecular modelling. R.L. and S.G. carried out the safety studies. S.W., M.T., S.P., M.D.R., R.L., S.G., M.D.U., L.M.M., F.Z., M.B., D.G., W.D.G., S.D., J.M.F., P.W.G., M.A.J.F., A.H.F., T.J.M., K.D.R. and I.H.G. designed experiments, managed parts of the project and contributed to the writing. See Supplementary Information for further details.

**Competing interests**

These authors have shared in GlaxoSmithKline: P.G.W., S.D., T.J.M., K.D.R., S.C., R.L., S.G., M.B., H.P., C.D., D.G., D.S.-D. and J.M.F. The other authors declare no competing interests.

**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s41586-018-0356-z.

**Supplementary information**

is available for this paper at https://doi.org/10.1038/s41586-018-0356-z.

**Reprints and permissions information**

is available at http://www.nature.com/reprints.

**Correspondence and requests for materials**

should be addressed to T.J.M., K.D.R. or I.H.G.

**Publisher’s note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
Extended Data Fig. 1 | Rate-of-kill of *L. donovani* axenic amastigotes by compound 7. Chart shows relative luminescence units (RLU) versus time from axenic amastigote rate-of-kill experiment with compound 7 (representative results for one of two independent experiments are shown; data are mean and s.d. of three technical replicates). Concentrations are as follows (μM): 50, open circles; 16.7, closed circles; 5.6, open squares; 1.85, closed squares; 0.62, open triangles; 0.21, closed triangles; 0.069, open inverted triangles; 0.023, closed inverted triangles; 0.0076, open diamonds and 0.0025, closed diamonds.
Extended Data Fig. 2 | Linker-containing target molecules synthesized for chemical proteomic experiments and their corresponding EC$_{50}$ values. Potencies of the compounds in the cidal axenic and intra-macrophage assays are shown; data are from at least three independent replicates.
Extended Data Table 1 | Activity of compound 7 and miltefosine against a panel of Leishmania clinical isolates

| Strain               | Country of origin | Year | Compound 7 EC$_{50}$ (µM) | Miltefosine EC$_{50}$ (µM) |
|----------------------|-------------------|------|---------------------------|-----------------------------|
| *L. donovani* LV9    | Ethiopia          | 1967 | 0.05, 0.09                | 0.36, 0.43                  |
| *L. donovani* SUKA 001 | Sudan             | 2010 | 0.09                      | 0.91                        |
| *L. donovani* BHU1 * | India             | 2002 | 0.11                      | 0.50                        |
| *L. donovani* DD8    | India             | 1980 | 0.13                      | 0.51                        |
| *L. infantum* ITMAP263 | Morocco           | 1967 | 0.50, 0.13                | 1.0, 0.79                   |

Intra-macrophage assays using human peripheral blood mononuclear cells are shown. Strains were tested as technical duplicates on a single (DD8, SUKA 001, BHU1) or on two (LV9, ITMAP263) occasion(s); the respective EC$_{50}$ values are shown for LV9 and ITMAP263. References for the cell lines are given in the Supplementary Information.

*Antimony-resistance reference strain.*
Extended Data Table 2 | Solubility of compound 7 in simulated physiological media

| Media       | Final pH | Solubility [mg/mL] |
|-------------|----------|--------------------|
| SGF pH1.6   | 1.5      | 1.12               |
| Fasted SIF pH6.5 | 6.5    | 0.017              |
| Fed SIF pH6.5 | 6.5     | 0.025              |

SGF, simulated gastric fluid; SIF, simulated intestinal fluid. Data were generated using crystalline polymorph form 1. Solubility experiments were performed at 37 °C for 4 h.
Extended Data Table 3 | In vitro metabolic stability data for compound 7

| Species | Concentration (µM) | Liver Microsomes Cl\text{\textsubscript{i}} (mL/min/g tissue) | Hepatocytes Cl\text{\textsubscript{i}} (mL/min/g tissue) |
|---------|-------------------|------------------------------------------------------------|----------------------------------------------------------|
| Mouse   | 0.5               | 0.52                                                       | 0.84                                                     |
| Rat     | 0.5               | <0.5                                                       | 0.77                                                     |
| Dog     | 0.5               | <0.4                                                       | 0.31                                                     |
| Human   | 0.5               | 0.71                                                       | 0.55                                                     |

Cl\text{\textsubscript{i}}, intrinsic clearance.
Extended Data Table 4 | Drug metabolism and pharmacokinetics data for compound 7

|          | Mouse (male, CD1) | Rat (male, SD) |
|----------|-------------------|----------------|
|          | 1 mg/kg           | 1 mg/kg        |
| Cl (ml/min/kg) | 169 ± 50          | 14 ± 9         |
| V_{dss} (L/kg) | 4.0 ± 0.5         | 0.4 ± 0.1      |
| T_{1/2} (h)   | 0.3 ± 0.04        | 0.4 ± 0.2      |
| AUC_{(0-inf)} (ng.h/mL) | 104 ± 26 | 1514 ± 782 |
| Oral      | 10 mg/kg          | 10 mg/kg       |
| C_{max} (ng/mL) | 561 ± 148         | 1043 ± 261    |
| T_{max} (h)  | 2                 | 2              |
| AUC_{(0-inf)} (ng.h/mL) | 1463 ± 362 | 6475 ± 2494 |
| F% based on AUC_{(0-inf)} | >100             | 46 ± 18       |
| Oral      | 100 mg/kg         | 100 mg/kg      |
| C_{max} (ng/mL) | 8813 ± 1966       | 8470 ± 3750   |
| T_{max} (h)  | 3                 | 7.3            |
| AUC_{(0-inf)} (ng.h/mL) | 39433 ± 23830 | 61202 ± 23591 |
| F% based on AUC_{(0-inf)} | >100             | 40 ± 15       |
| Oral      | 300 mg/kg         | 300 mg/kg      |
| C_{max} (ng/mL) | 11393 ± 4212      | 14833 ± 2676  |
| T_{max} (h)  | 5                 | 7.3            |
| AUC_{(0-inf)} (ng.h/mL) | *66150 ± 636   | 136333 ± 24846 |
| F% based on AUC_{(0-inf)} | >100             | 51 ± 22       |

AUC, area under the curve; Cl, clearance; C_{max}, maximum concentration; F%, oral bioavailability; V_{dss}, volume of distribution at steady-state; T_{1/2}, half-life; T_{max}, time at which maximum concentration is reached. CD1 mice and Sprague Dawley (SD) rats were used. Each arm was carried out with three animals.

*Back-extrapolated AUC greater than 20%.
## Extended Data Table 5 | Sensitivity of wild-type and drug-resistant promastigotes to compounds within the series

| Cell line                        | Compound 4 |   | Compound 5 |   | Compound 7 |   |
|---------------------------------|------------|--|------------|--|------------|--|
|                                 | pEC₅₀ (s.d.) | Fold | pEC₅₀ (s.d.) | Fold | pEC₅₀ (s.d.) | Fold |
| Wild type (Start clone)         | 7 (0.1)    | 1   | 8.2 (0.4)  | 1   | 7.1 (0.3)  | 1   |
| Wild type (Age-matched)         | 7.1 (0.2)  | 1   | 8.2 (0.1)  | 1   | 7.3 (0.2)  | 1   |
| 4-resistant clone 1             | < 4.3      | >500 | 7.2 (0.1)  | 11  | 5.8 (0.4)  | 20  |
| 4-resistant clone 2             | < 4.3      | >500 | 7.3 (0.1)  | 7   | 5.7 (0.2)  | 24  |
| 4-resistant clone 3             | < 4.3      | >500 | 7 (0.2)    | 17  | 5.4 (0.1)  | 48  |
| 5-resistant clone 1             | < 4.3      | >500 | 7.1 (0.2)  | 11  | 5.5 (0.2)  | 41  |
| 5-resistant clone 2             | < 4.3      | >500 | 7.1 (0.2)  | 14  | 5.5 (0.1)  | 35  |
| 5-resistant clone 3             | < 4.3      | >500 | 7.3 (0.1)  | 9   | 5.7 (0.1)  | 22  |

Resistance was generated against compounds 4 and 5. Values in parentheses denote s.d., n = 3 independent replicates. pEC₅₀, negative logarithm of the EC₅₀ value. Potencies are mean pEC₅₀.
### Extended Data Table 6 | Sensitivity of wild-type and compound 5-resistant intra-macrophage amastigotes to the compound series

| Compound | Cell line | pEC$_{50}$ | Host cell pEC$_{50}$ | Fold difference |
|----------|-----------|------------|----------------------|-----------------|
| 5        | WT        | 7.5        | <5.3                 | -               |
| 5        | 5 RES clone 1 | 6.6        | <5.3                 | 8.5             |
| 7        | WT        | 5.9        | <4.3                 | -               |
| 7        | 5 RES clone 1 | 5.2        | <4.3                 | 5.0             |

RES, resistant; pEC$_{50}$, negative logarithm of the EC$_{50}$ value (the compound concentration showing 50% inhibition of the growth of the cells).
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed |
|-----|-----------|
| ☒  | The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☒  | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☒  | The statistical test(s) used AND whether they are one- or two-sided |
| ☒  | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| ☒  | A description of all covariates tested |
| ☒  | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☒  | A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☒  | For null hypothesis testing, the test statistic (e.g. \(F\), \(t\), \(r\)) with confidence intervals, effect sizes, degrees of freedom and \(P\) value noted |
| ☒  | Give \(P\) values as exact values whenever suitable. |
| ☒  | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☒  | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☒  | Estimates of effect sizes (e.g. Cohen’s \(d\), Pearson’s \(r\)), indicating how they were calculated |
| ☒  | Clearly defined error bars |
| ☒  | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

Perkin Elmer Columbus 2.8.1 was used for image analysis (parasite and host cell counting) used in the intramacrophage assay.

Data analysis

Compound potencies against Leishmania parasites infecting macrophages were calculated in IDBS Activitybase using a 4-parameter logistic equation. Compound potencies of drug-resistant Leishmania promastigotes were calculated by non-linear regression in GraFit using a 4-parameter logistic equation. The SILAC analysis used MaxQuant 1.5.2.8 and Perseus 1.3.0.4 (http://www.coxdocs.org). Other proteomic analysis used Mascot (Matrix Science) and IsobarQuant. Molecular modeling used modules from SCHRODINGER, including GLIDE. Molecular dynamics was carried out using AMBER14.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data
Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The raw sequence data are available at the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the study PRJEB583 - this statement is put in section 4.13 in the supporting information where the experiment is described. Proteomic data is archived in the PRIDE database with the dataset identifiers PXD009711 and PXD09764. The statements for this are in sections 4.4 and 4.5

Field-specific reporting
Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [ ] Life sciences
- [ ] Behavioural & social sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences

Study design
All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes are given in the manuscript. Efficacy studies in mice were conducted with 5 mice per group |
|-------------|------------------------------------------------------------------------------------------------------|
| Data exclusions | No data was excluded. |
| Replication | Replication numbers are reported in the text or in the methodology sections. Where appropriate a measure of the error is reported. |
| Randomization | Infected mice for the efficacy studies were randomly allocated into experimental groups. |
| Blinding | For the mouse efficacy studies, the same investigators carried out the animal infection, dosing of test compound and analysis so were not blinded to group allocation |

Materials & experimental systems

Policy information about availability of materials

| n/a | Involved in the study |
|-----|-----------------------|
| [x] | Unique materials |
| [ ] | Antibodies |
| [x] | Eukaryotic cell lines |
| [ ] | Research animals |
| [x] | Human research participants |

Unique materials

Obtaining unique materials
Compound 7 is currently in pre-clinical development and full disclosure of the synthesis of this compound has been included in this publication. All reasonable requests for the other key tool molecules disclosed in this manuscript will be met subject to an appropriate material transfer agreement in place between all parties.

Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | THP-1 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) |
|---------------------|------------------------------------------------------------------------------------------------|
| Authentication | ECACC certifies authenticity of their cell lines. |
| Mycoplasma contamination | THP-1 cells were mycoplasma tested and confirmed negative. |
| Commonly misidentified lines (See ICLAC register) | Name any commonly misidentified cell lines used in the study and provide a rationale for their use. |
Research animals

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Animals/animal-derived materials

Mouse: CD1, male, adult; BALB/c, female, adult; Rat: Sprague Dawley, male, adult; Wistar, male, adult and Hamster, male, Golden Syrian, adult

Method-specific reporting

n/a | Involved in the study

- [x] ChIP-seq
- [ ] Flow cytometry
- [x] Magnetic resonance imaging

Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

| Sample preparation | Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used. |
|--------------------|------------------------------------------------------------------------------------------------------------------|
| Instrument         | Identify the instrument used for data collection, specifying make and model number.                                  |
| Software           | Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details. |
| Cell population abundance | Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined. |
| Gating strategy    | Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between "positive" and "negative" staining cell populations are defined. |

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.