Title: DNDI-6174 is a preclinical candidate for visceral leishmaniasis that targets the cytochrome *bc₁* complex

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One sentence summary: We report the characterization of a *Leishmania* cytochrome *bc*$_1$ complex inhibitor with potential for treating leishmaniasis.

Abstract: New drugs for visceral leishmaniasis that are safe, low cost and adapted to the field are urgently required. Despite concerted efforts over the last several years, the number of new chemical entities that are suitable for clinical development for the treatment of *Leishmania* remains low. Here, we describe the discovery and preclinical development of DNDI-6174, an inhibitor of *Leishmania* cytochrome *bc*$_1$ complex activity that originated from a phenotypically-identified pyrrolopyrimidine series. This compound fulfills all target candidate profile criteria required for progression into preclinical development. In addition to good metabolic stability and pharmacokinetic properties, DNDI-6174 demonstrates potent *in vitro* activity against a variety of *Leishmania* species and can reduce parasite burden in animal models of infection, with the potential to approach sterile cure. No major flags were identified in preliminary safety studies, including an exploratory 14-day toxicology study in the rat. DNDI-6174 is a cytochrome *bc*$_1$ complex inhibitor with acceptable development properties to enter preclinical development for visceral leishmaniasis.

Introduction

Leishmaniasis is a complex vector-borne disease in which parasites are transmitted through the bite of female phlebotomine sandflies. Associated with poverty and classified as a neglected infectious disease, leishmaniasis is one of the diseases targeted in the United Nations Sustainable Development Goals (SDGs) agenda, specifically SDG3.3 (*1*). There are more than 20 causative species and various manifestations in different regions of the world. These range from visceral leishmaniasis (VL), also known as kala-azar, a systemic disease that is fatal if left untreated, to cutaneous leishmaniasis (CL). In Asia and East Africa, VL is mostly caused by *Leishmania donovani* and the disease is anthroponotic, whereas it is caused by *L. infantum* in Latin America and the Mediterranean region where the disease is zoonotic, with the dog being the main reservoir. Additionally, painless post-VL skin lesions, (which contribute to continued disease transmission), post-kala-azar dermal leishmaniasis (PKDL) and muco-cutaneous leishmaniasis (MCL) occur in some regions of the world (*2*). Patients with HIV/VL co-infection present a further complicated form of the disease (*2*). Worldwide, 1 in 7 people live in leishmaniasis-endemic areas with an estimated 1 billion people at risk of infection from VL and CL. The disease is highly endemic in the Indian subcontinent and in East Africa, with more than 90% of new cases reported to WHO occurring in 7 countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan, with a high rate of underreporting. Every year, there are between 50,000-90,000 new cases of VL responsible for 20,000-30,000 deaths (*2*). Currently, the region with the highest burden worldwide is Eastern Africa, with most cases observed in Ethiopia, Kenya, Somalia, Sudan, South Sudan and Uganda.

Historically, antimonial monotherapy (such as sodium stibogluconate, SSG) for 20-30 days has been the mainstay treatment for VL. In the last 15 years, efforts have been made to develop better and safer treatments with liposomal amphotericin B, followed by paromomycin (PM) and miltefosine (MIL), being made available for use. Liposomal amphotericin B is

currently considered as the standard treatment for the elimination of VL in India. However, this drug is not as effective in East Africa, and pentavalent antimonials remain a component of the primary first-line treatment in that area, with numerous drawbacks in terms of the parenteral route of administration, length of treatment, toxicity, and cost. A single first-line treatment (SSG and PM), which has safety concerns, combined with poorly effective second-line treatments that are difficult to administer, are not sufficient for elimination of this disease. The priority is therefore to deliver an anti-parasitic drug that will meet the Target Product Profile (TPP) for VL published by Drugs for Neglected Diseases initiative (DNDi) (*3*) and hence provide an orally active, safe, effective, short-course and field-adapted treatment for leishmaniasis that would have the potential to revolutionize treatment. Indeed, simplification and shortening of the dosing regimen with the development of oral drugs for VL would certainly improve treatment access and compliance, reduce substantial side effects of the current treatments and reduce the potential emergence of resistance, thereby contributing to the SDG goals towards elimination of the disease by 2030. DNDi through its Drug Combination Development Platform (DCDP) aims to develop a combination therapy based on newly developed New Chemical Entities (NCEs) to achieve short course therapy with satisfactory efficacy having utility for all regions with disease and counter the potential emergence of drug resistance. This would improve and simplify current case management and support elimination efforts. The current pipeline of oral NCEs for VL at the translational stage is unprecedented but the risk of attrition remains high. Hence, anticipation of a high rate of attrition in NCE development is a compelling argument to continue efforts to add to the pipeline.

Here we report the discovery of DNDI-6174 and present data to support its selection as a preclinical development candidate for VL. DNDI-6174 originated from a potent hit discovered following a phenotypic screening campaign conducted by GSK (*4*) and was optimized via a medicinal chemistry program focused initially on a related kinetoplastid

parasite, *Trypanosoma cruzi* (*T. cruzi*); compounds from this series proved valuable for combination treatments for Chagas disease (*5*). DNDI-6174 belongs to a chemical class of pyrrolopyrimidines and targets the Q_i site of *Leishmania* cytochrome *b*, leading to inhibition of cytochrome bc_1 complex (III) activity. Earlier work conducted by Wellcome in the 1980's and 90's established the activity of a series of hydroxynaphthoquinones against the same target (*6,7*), however the early series were plagued by issues of cytotoxicity and poor oral bioavailability precluding their further development. With pharmacological, physicochemical and pharmacokinetic properties that support short term oral administration consistent with DNDi's Target Candidate Profile (TCP) for VL, DNDI-6174 is therefore a candidate with suitable development properties to reach the preclinical development stage.

Results

Identification and optimization of a promising pyrrolopyrimidine

High-throughput screening of GSK's 1.8M compound library against *L. donovani*, *T. cruzi* and *T. brucei* resulted in the identification of a number of compounds active against these parasites (*4*). Among these hits, TCMDC-143610 (Fig. **1**) demonstrated promising activity against *T. cruzi*, (EC_{50} value of 130 nM), and modest activity against *L. donovani* with an EC_{50} value of 15.8 µM against the intracellular amastigote stage of this parasite. A medicinal chemistry program focused on developing compounds targeting *T. cruzi* successfully delivered potent, advanced leads with appropriate pharmacokinetic profiles. Leading compounds were profiled *in vitro* and *in vivo* against *Leishmania spp.* revealing encouraging activity against this parasite as well.

A *Leishmania*-focused lead optimization program was subsequently undertaken to optimize potency, safety, physicochemical and pharmacokinetic properties. The details of the medicinal chemistry program and structure activity relationships (SAR) that led to the discovery of DNDI-0003366174 (aka DNDI-6174) will be described in a separate publication.

In vitro profiling of DNDI-6174

The potency of DNDI-6174 was established against a broad range of *Leishmania* species and isolates where it was found to be a potent inhibitor of *L. donovani* and *L. infantum*, the etiological agents of VL and was also active against several species that cause CL (Table **1**). DNDI-6174 was also found to be broadly active against a panel of representative VL clinical isolates from various sources and importantly, it remained active against clones resistant to currently used therapeutics such as MIL and PM (Table **1**).

DNDI-6174 was also profiled for its activity against other disease-causing kinetoplastid parasites. While demonstrating impressive potency against *T. cruzi* (Tulahuen strain, EC_{50} value < 5 nM), the causative agent of Chagas disease, the compound was considerably less active against *T. brucei brucei* and *T. b. rhodesiense* (bloodstream form) associated with human African trypanosomiasis with EC_{50} values ranging from 5 to 20 µM.

Efficacy of DNDI-6174 in animal models of VL

Given its promising *in vitro* profile, the efficacy of DNDI-6174 was evaluated in mouse (*L. donovani* or *L. infantum* infection) and hamster (*L. infantum* infection) models of VL. DNDI-6174 administered for 5 consecutive days at doses of 12.5 mg/kg twice daily (bid) or 25 mg/kg once daily (qd) resulted in a >98% decrease in the liver parasite burden of infected BALB/c mice compared to control animals (dosed with vehicle). Similar efficacy was observed when the dose was reduced to 6.25 mg/kg bid and the duration of treatment was extended to 10 days. Assessment of 5-day dosing regimens in mice infected with either *L. infantum* or *L. donovani* produced similar results. Indeed, efficacy (>95% reduction in liver parasite burden) was reached at a dose of 12.5 mg/kg bid or higher, while 6.25 mg/kg bid was not sufficient for either species with a 5-day dosing duration. A summary of results obtained following different dose and treatment durations is shown in Table **S1A**.

To fully appreciate the potential of DNDI-6174 as a drug candidate for VL, the compound was assessed in hamsters (*L. infantum* only) as a complementary model to the mouse VL model, allowing parasite load evaluation in all major target organs (liver, spleen and bone marrow). Once daily dosing at and above 12.5 mg/kg showed excellent efficacy, leading to a reduction of the parasite burden of more than 99% in all three organs (Table **S1B**). The promastigote back-transformation assay was used to qualitatively assess the potential for sterile cure. Here, the organs of treated animals were recovered, macerated, and introduced to *in vitro* culture (*8*). Inoculated cultures were then monitored for the emergence of viable promastigotes. In DNDI-6174-treated animals, almost all the organs recovered proved to be negative in culture (Table **S2**) thus suggesting its potential to approach sterile cure *in vivo*. While many organs were negative in the back-transformation, 2/5 hamsters were still positive in the highest DNDI-6174 dosing group in at least one organ, indicating that sterile cure was not achieved in all animals. In contrast, all organs tested in the vehicle control group were positive, and more than half of those following miltefosine administration at 40 mg/kg were positive. Back-transformation results (Table **S2**) corroborated the Giemsa data (Table **S1**) demonstrating the superiority of the 5-day DNDI-6174 treatment at 12.5 mg/kg bid to the 5-day miltefosine administration at 40 mg/kg qd. A once daily administration of 6.25 mg/kg DNDI-6174 was not sufficient for efficacy while bid dosing at 6.25 mg/kg resulted in more than 95% reduction in parasite burden. Consequently, the minimum efficacious daily dose of DNDI-6174 in the hamster model was estimated to be 12.5 mg/kg given qd for 5 days or 6.25 mg/kg given bid for 5 days.

Mechanism of action studies

Cross-resistance profiling

As a first step towards determining the mechanism of action (MoA), DNDI-6174 was screened against a panel of *L. donovani* promastigote cultures that are resistant to drugs and compounds with defined mechanisms of action and molecular targets. In this screen, three independent clonal cell lines resistant to DDD01716002, an established inhibitor of the cytochrome bc_1 complex of the electron transport chain (ETC) (*9*), demonstrated considerable (3.4 – 34-fold) cross-resistance to DNDI-6174 (Table **2** and Fig. **2A**).

DDD01716002 is a specific inhibitor of the Q_i site of cytochrome *b* exploiting the same hydrophobic pocket as the well-established Q_i site inhibitor antimycin A (*9*). Cell lines resistant to this compound bear mutations within this region of the enzyme (summarized in Table **2**).

Resistance generation and whole-genome sequencing

L. donovani promastigote cultures resistant to DNDI-6174 were generated by culturing clonal, drug-susceptible parasites in the continuous presence of compound *in vitro* (Fig. **2B**). *L. donovani* promastigotes exposed to increasing concentrations of compound over a 25-day period and then cloned by limiting dilution were between 6- and 164-fold less susceptible to DNDI-6174 than the WT parental line (Table **2**). In each case the resistance demonstrated by all five of the clones was stable over 20 passages in the absence of compound selection.

Whole genome sequencing (WGS) revealed that all five DNDI-6174-resistant clonesmaintained mutations within the gene encoding cytochrome *b* (Fig. **2C** and Table **2**). All mutations were found to map to the ubiquinone reduction center of cytochrome *b* (Q_i site) and notably two were identical to mutations previously identified in parasites resistant to the established Q_i inhibitor DDD01716002 (Gly31Ala and Ser207Pro) (*9*). Analysis of sequencing indicates that all cytochrome *b* mutations are homozygous. Outside of these mutations, no other common single nucleotide polymorphisms (SNP) or copy number variations (CNV) were identified in the genomes of our drug resistant clones (Table **S3**, Table **S4**, and Fig. **S1**).

Cytochrome *b* is encoded solely by the maxi-circle DNA, a minor component of the parasite's kinetoplast DNA (*11*), equivalent to mitochondrial DNA in mammalian cells. These mitochondrial DNA networks can maintain up to 50 copies of maxicircle DNA meaning that a single network can encode up to 50 copies of *cytochrome b*. This and other factors effectively preclude investigating the role of specific cytochrome *b* mutations in resistance by genetic methods. Nevertheless, these data strongly suggest that mutations within cytochrome *b* are driving resistance to DNDI-6174 and indicate that Q_i site of cytochrome *b* is the likely target of this promising compound.

DNDI-6174 inhibits complex III activity

To establish if DNDI-6174 specifically inhibits complex III activity in *L. donovani*, clarified cell lysates that were enriched in mitochondria were prepared. Complex III activity was monitored in the presence and absence of DNDI-6174 using decylubiquinol as a pseudo-substrate, as previously described (*9*). As expected, DNDI-6174 proved to be a potent inhibitor of complex III in lysates prepared from both promastigote and axenic amastigote stages of *L. donovani*, returning IC_{50} values of 8 ± 1.7 and 2 ± 0.5 nM, respectively ($n \geq 3$ biological replicates) (Table **3**). These values correlate with EC_{50} values for DNDI-6174 against promastigotes and axenic amastigotes of 24 ± 1 and 75 ± 2 nM, respectively ($n \geq 3$ biological replicates) and support our hypothesis that cytochrome *b* is the principal target of this compound.

Modelling

To gain insight into the binding mode of DNDI-6174 to *L. donovani* cytochrome *b* and to rationalize the role of mutations associated with resistance, we carried out molecular modelling studies. Our previously established *L. donovani* cytochrome *b* model was used as a starting point for these studies (*9*). In the first instance, DNDI-6174 was docked into the Q_i site of WT

cytochrome *b*. In the most favorable binding pose, the 2-aminopyrrolopyrimidine scaffold fits well into the proposed binding site and is stabilized by polar interactions with Asp231 and a water-mediated H-bond with the backbone of Phe34 (Fig. **3,** Fig. **S2 A**-**B**). Multiple π-π stackings are established both by the heterocyclic scaffold and the benzodioxole moiety with Phe223 and His202, respectively.

Our *L. donovani* cytochrome *b* model was then used in molecular dynamics simulations at 100 ns. These studies provided a rationale for the impact of resistance conferring mutations. DNDI-6174 was docked into models of the five mutated enzymes. In all cases, DNDI-6174 was accommodated into the Q_i binding site of cytochrome *b* in a similar manner to that seen for the WT enzyme, with no major clashes observed (Fig. **S2**). However, these mutations are predicted to impact the binding of DNDI-6174 in several ways, principally by reducing the stability of target-ligand interactions. A detailed description of the effects of individual mutations on ligand binding is provided in supplementary information (Fig. **S3-8**).

Physicochemical, permeability and binding properties of DNDI-6174

Physicochemical, permeability and binding properties for DNDI-6174 are shown in Table **S5**. DNDI-6174 is characterized as having a relatively low molecular weight, moderate lipophilicity, and low solubility in physiologically relevant biological media. Binding of DNDI-6174 to plasma proteins from various species is moderate.

The permeability of DNDI-6174 across Caco-2 cell monolayers was found to be high with an efflux ratio of approximately 1.0. The effective human jejunal permeability (P_{eff}) was predicted using the measured Caco-2 apparent permeability coefficient (A-B P_{app}) and the previously described relationship between Caco-2 permeability and P_{eff} (*12*) determined using the same experimental conditions. The predicted P_{eff} is high (5.1 x 10^{-4} cm/s) suggesting that permeability is not likely to limit oral absorption in humans. Based on the DCS classification

system (*13*), the solubility limited absorbable dose (SLAD) is approximately 35 mg indicating that solubility within the intestine could limit oral absorption if the human dose is high and formulation approaches may be needed to address this issue.

Nonclinical metabolism of DNDI-6174

The metabolism of DNDI-6174 was assessed *in vitro* using liver microsomes and cryopreserved hepatocytes from mice, rats, dogs and humans. Intrinsic clearance and predicted *in vivo* hepatic plasma clearance (factoring in binding to the *in vitro* test system, plasma proteins, and the blood to plasma ratio) are shown in Table **S6**. Overall, microsomes appeared to give better predictions of the *in vivo* clearance in dogs, rats and mice compared to hepatocytes, assuming clearance occurs predominantly by hepatic metabolism (see subsequent pharmacokinetic sections). Based on these predictions, DNDI-6174 would be expected to have low plasma clearance (~1 mL/min/kg) in humans.

Further studies were conducted to determine the major metabolites of DNDI-6174 using *in vitro* test systems (liver microsomes and hepatocytes) and comparison of the chromatographic retention and MS/MS fragmentation characteristics to authentic standards for the proposed metabolites. The results are summarized in Fig. **4A.** In liver microsomes, three primary metabolites were detected, corresponding to two oxygenation products having molecular ions 16 amu higher than the parent compound (designated M+16 (I) and M+16 (II)) and a product with a molecular ion 12 amu lower than the parent (designated M-12). In addition to these three primary metabolites, two secondary metabolites having masses of 34 amu higher than the parent were also detected. Qualitatively, the same metabolites were formed in human, dog and rat microsomes. Two potential metabolites were synthesized (DNDI0003671146 and DNDI0003578765, Fig. **4A**) and were confirmed to be metabolites M+16 (I) and M-12, respectively; both were found to be inactive against *Leishmania*. Three other potential

metabolites were also synthesized (DNDI0003671392, DNDI0003933419 and DNDI0003947637, Fig. **4B**), however, none of these were detected in either the microsome or hepatocyte incubations. For incubations with hepatocytes, several secondary products were seen that corresponded to glucuronide conjugates of M-12 (designated M+164), of M+16 (I) (designated M+192 (I)) and of M+16 (II) (designated M+192 (II)) (Fig. **4A**). The identity of M+16 (II) has yet to be confirmed but based on the potential metabolites that can be excluded and CID fragmentation patterns, it is thought to result from *N*-oxygenation of one of the pyrrolopyrimidine nitrogens.

Reaction phenotyping studies with chemical inhibitors were conducted using human liver microsomes to determine the main cytochrome P450 (CYP) enzyme(s) involved in the primary metabolism of DNDI-6174. There were no significant differences in the CL_{int} values in the absence or presence of inhibitors for any of the isoforms tested, however, there was a non-significant trend for a reduction in CL_{int} in the presence of CYP1A2 and CYP3A4/5 inhibitors suggesting a potential contribution of these enzymes (Fig. **S9**). By monitoring metabolite formation (Fig. **S10**) it was clear that M+16 (I) is formed by CYP3A4/5 and CYP2B6, and M+16 (II) is formed by CYP1A2. M-12 appeared to be formed by a combination of CYP3A4/5 and CYP2C8. Using the available authentic metabolite standards, only about 75% of the initial DNDI-6174 could be accounted for by the remaining parent and the known primary metabolites, suggesting the presence of additional, yet to be identified minor metabolites (e.g. M+16 (II) and possibly others).

Nonclinical pharmacokinetics of DNDI-6174

The *in vivo* pharmacokinetic properties for DNDI-6174 were characterized in female BALB/c mice, male Sprague Dawley rats, male beagle dogs and female golden Syrian hamsters. Following intravenous administration (Table **S7** and Fig. **5**), the compound had low plasma

clearance, a moderate volume of distribution and moderate half-life across species. In rats, <1% of the parent compound was excreted unchanged in urine, suggesting that hepatic metabolic clearance is the major route of elimination.

Data following single oral administration in mice, rats, dogs and hamsters (day 1 of a multiple dose regimen for hamsters) are also shown in Table **S7** and Fig. **5**. The oral apparent bioavailability in mice was high and exceeded 100% at 25 mg/kg indicating possible saturation of first-pass and/or systemic clearance. It should also be noted that plasma concentrations after IV dosing in mice were only measurable up to 7.5 h and the terminal phase was not well defined, which will lead to error in the IV area under the concentration versus time curve (AUC) estimation. In rats, oral bioavailability was also high and the maximum concentration (C_{max}) and AUC increased in proportion to the increase in dose between 10 and 50 mg/kg (Table **S8**) but dropped off as doses increased up to 300 mg/kg. In dogs, single dose exposure appeared to increase to a greater extent than the increase in dose between 5 and 30 mg/kg, but then decreased relative to dose from 30 to 90 mg/kg (Table **S9**). Intravenous dosing in hamsters was not conducted and therefore bioavailability could not be calculated for this species.

Following bid dosing in mice (Figure **S11A**), a marginal increase in C_{max} (approximately 2-fold) was observed, but AUC (0-24 h and 96-120 h) remained relatively constant over the 5-day dosing period (Table **S10**). In hamsters (Figures **S11B**), both C_{max} and AUC increased over the dosing period following qd dosing for 5 days (Table **S11**).

One-compartmental fits of the mouse concentration versus time data were generally good and the error estimates for the primary parameters were generally less than 20% (Table **S12**). Error estimates for the secondary parameters were less than 15% at all dose levels. There was more variability in the hamster profiles, which led to greater error in the estimated primary parameters. Error estimates for the secondary parameters were less than 15% at all but the lowest hamster dose.

Fitted data for AUC over 24 h at steady state and C_{max} at steady state as a function of dose are shown in Fig. **S12** along with the experimental data on days 1 and 5 of dosing. Note that AUC_{24} at steady state is equivalent to $AUC_{0-\infty}$ for a single dose and twice $AUC_{0-\infty}$ for twice daily dosing, assuming linear kinetics. On balance, the fits provide an estimation of the profiles at each dose level and species and were considered suitable for simulating repeat dose profiles under the conditions used in the efficacy studies where exposure could not be assessed for practical reasons.

PK/PD analysis and human dose prediction

To determine the correlation between the pharmacokinetic (PK) and pharmacodynamic (PD) data, repeat dose profiles in mice and hamsters following single and twice daily dosing were simulated using the fitted parameters. A necessary assumption was made that the PK properties were comparable in healthy and diseased animals. Simulated profiles are shown in Fig. **S13**. Pharmacodynamic data in mice and hamsters infected with *L. infantum* or *L. donovani* and in hamsters infected with *L. infantum* and treated with DNDI-6174 are tabulated along with the extracted plasma parameters in Table **S13** and Table **S14**, respectively.

As shown in Fig. **6,** higher cumulative exposure (unbound AUC) was required in mice compared to hamsters to achieve 95% reduction in liver burden (Table **S15**). Other parameters were less well correlated to efficacy, presumably given that none reflect the cumulative exposure profile with an extended treatment regimen. Based on these results, the cumulative unbound exposure (AUC) required for 95% reduction in liver burden relative to control is approximately 4 μg.h/mL based on data in hamsters and approximately 27 μg.h/mL based on mice. Results for mice infected with *L. donovani* were similar to those for *L. infantum* (Table **S15**).

Human plasma clearance was estimated by *in vitro/in vivo* extrapolation (IVIVE) of data from liver microsomes and allometry using the available *in vivo* preclinical data. In both cases, renal excretion of unchanged drug was assumed to be negligible (experimentally confirmed in the rat). For IVIVE, the geometric mean of the error in the predicted unbound intrinsic clearance across the three preclinical species (1.4-fold underprediction) was used as a correction factor for predicting the human unbound intrinsic clearance as described previously (*14*). A body weight of 50 kg for the patient population was used giving a predicted human plasma clearance of 1.15 mL/min/kg (3.45 L/h). Simple allometry of unbound clearance from mice, rats and dogs was found to be unsuitable for the prediction of human clearance as the allometric exponent was high (>1.0). Therefore, the "rule of exponents" was applied and the unbound clearance data were corrected for brain weight (*15*) as described previously (*16*). Using this approach, the predicted human clearance (again assuming a body weight of 50 kg) was 0.90 mL/min/kg (2.71 L/h). The mean of these two values therefore gives a final predicted human clearance of 1.03 mL/min/kg (3.08 L/h)

The predicted clearance was input along with the data in Table **S16** into a physiologically-based pharmacokinetic (PBPK) model (GastroPlus) to simulate human profiles under different dosing conditions. Given that there was no evidence of renal elimination in rats, it was assumed that clearance occurs solely by hepatic metabolism. The model predicted half-life and volume of distribution in humans (50 kg body weight) are 12.0 h and 1.1 L/kg, respectively.

Human PK profiles were then simulated to determine the dose and dose regimen necessary to achieve the target exposure profile based on data in hamsters and mice (cumulative unbound plasma AUC of 4 and 27 µg.h/mL, respectively equivalent to a cumulative total human plasma AUC of 60 and 460 µg.h/mL, Fig. **S14**). For a 10-day dosing period, the

estimated human dose is 20-180 mg (qd) or 10-80 mg (bid). For a 14-day dosing period, the estimated dose is 15-120 mg (qd) or 7.5-55 mg (bid).

Early safety profiling of DNDI-6174

In vitro de-risking was performed to identify any potential cardiotoxicity, cytotoxicity and mutagenicity liabilities that could preclude the clinical development of DNDI-6174. *In silico* and *in vitro* risk assessment of cardiotoxicity predicted little to no change in QT interval at exposures up to 30 μM but a profile possibly associated with an effect on hemodynamics at high exposures (Table **S17** and Fig. **S15**). In cell cytotoxicity (CC) assays, DNDI-6174 showed no activity (CC_{50} >37 μM) against all cell lines tested (Table **S18**). The AMES analysis of DNDI-6174 did not show intrinsic mutagenic potential in the absence or presence of rat liver S9 fraction for metabolic activation. In mammalian test systems (mouse lymphoma and CHO micronucleus), again in the absence or presence of rat liver S9, DNDI-6174 was not genotoxic.

DNDI-6714 did not show any activity in panels of mammalian receptor, enzyme or ion channel assays (Table **S19**), with the exception of alpha1A Human Adrenoceptor GPCR (α1A antagonism) and the human phosphodiesterases PDE3A and PDE4D2 for which IC_{50} values of 12.8, 3.36 and 6.67 μM, respectively, were measured. These activities observed for PDEs were not considered as relevant as physiological levels of these enzymes are at the low nM range (concentrations of cAMP, cGMP).

Since DNDI-6174 targets the electron transport chain in the mitochondria of *Leishmania*, selectivity away from human cytochrome bc_1 and no host mitochondrial toxicity were important issues of concern. The assessment of DNDI-6174 across a full dose range for potential mitochondrial toxicity followed a 3-step cascade whereby specific criteria were defined in order to continue development of this compound (Table **S20**). A substantial window (>1000 fold) was observed between the human cytochrome bc_1 complex biochemical assay

(<20% inhibition at 200 μM, Table **S21**) and the effects on the *bc₁* complex of *Leishmania donovani*. In the MitoExpress® mitochondrial function assay, oxygen consumption was reduced at concentrations ≥66 μM in HepG2 cells, with a maximum response limited to 28% at the highest concertation tested (200 μM). Likewise in the Seahorse® mitochondrial stress test in HepG2 cells, only a small reduction in oxygen consumption rate (OCR Basal) was observed up to a maximum of 14% reduction at 200 μM; the minimum effect concentration (MEC) for OCR reduction was 95.6 μM and there were no changes in reserve capacity, extracellular acidification rate (ECAR), maximum capacity or ATP production. Compared to the free concentration of DNDI-6174 at the predicted therapeutic plasma C_{max} in humans, this provides a window of approximately 57-fold compared to the MitoExpress® no-effect concentration in HepG2 cells and approximately 187-fold compared to the Seahorse® OCR MEC. There was a reduction in the calcium loading capacity of isolated mitochondria with DNDI-6174 treatment (XC_{50} = 10 μM), suggesting potential for some mitochondrial activity at lower concentrations that warrants further consideration, however the overall pattern of mitochondrial data with DNDI-6174 does not suggest appreciable direct inhibition of mammalian cytochrome bc_1 complex.

The risk of DNDI-6174 being phototoxic was discarded according to the results generated in the *in vitro* 3T3 Neutral Red Uptake (NRU) phototoxicity assay.

An exploratory 14-day toxicology study was performed with DNDI-6174 at a daily dose of 30, 80 or 200 mg/kg. All rats tolerated oral administration of 30 and 80 mg/kg with no mortality or morbidity. The dose of 200 mg/kg led to early sacrifice of one female on day 5. Furthermore, most animals of this group showed clinical signs suggesting a lack of tolerability including a body weight decrease of >10% in males which correlated with a decrease in food consumption. At this highest dose, white blood cell count was also affected and associated with decreases in thymus and spleen weights, consistent with stress. Minimal increase in liver

enzyme markers (alanine aminotransferase and aspartate aminotransferase) and total bilirubin concentration occurred in males at 80 and 200 mg/kg/day, while increased liver weights occurred in females at 80 and 200 mg/kg/day. Higher thyroid/parathyroid gland weights were observed in males at and above 80 mg/kg/day. In the absence of adverse findings at 80 mg/kg, this dose has been set as the no observed adverse effect level (NOAEL) for DNDI-6174. The dose of 80 mg/kg in the rat resulted in a 9- to 33-fold higher exposure (based on the AUC from time zero to 24 hours post-dose at steady state ($AUC_{24,ss}$) of 756 and 865 µg.h/mL in males and females, respectively) compared to the steady state AUC needed for efficacy in the mouse or hamster.

Early drug-drug interaction risk assessment

DNDI-6174 exhibited moderate inhibition against CYP1A2 (IC_{50} of 5.1 µM) and CYP3A4/5 (IC_{50} of 12.3 µM) and minimal inhibition (IC_{50} >15 µM) of the remaining isoforms (Table **S22**). Experiments conducted with a pre-incubation in the absence and presence of the co-factor, NADPH, suggested time-dependent inhibition of CYP2D6 and CYP3A4/5, and to a lesser degree CYP2C8.

DNDI-6174 was also assessed against a panel of drug transporters at concentrations of 1 and 10 µM showing less than 50% inhibition of BCRP, BSEP, OAT1, OAT3 OCT1 and OCT2. Low but detectable inhibition was observed against OATP1B1 (IC_{50} = 22 µM) and OATP1B3 (IC_{50} = 16 µM), while substantial inhibition of MATE2-K (IC_{50} = 0.88 µM) and MATE1-HEK (IC_{50} = 2.1 nM) was measured.

Discussion

Following a phenotypic screening campaign conducted by GSK (*4*), we have optimized a series of pyrrolopyrimidines as a new chemical class for potential treatment of leishmaniasis and identified DNDI-6174 as a promising oral preclinical candidate. DNDI-6174 showed potent *in vitro* antileishmanial activity against a range of *Leishmania* species responsible for causing VL or CL, as well as current drug resistant species and clinical isolates. While this compound was found to be very potent against another parasite of the kinetoplastida, *T. cruzi*, DNDI-6174 was only marginally active against *T. brucei*.

Comprehensive MoA studies confirmed that DNDI-6174 employs a different mechanism of action compared to anti-leishmanials in clinical use as well as the other NCEs in the current global leishmaniasis portfolio, an important consideration for potentially combining drugs for a better therapeutic outcome. This pyrrolopyrimidine targets *Leishmania* cytochrome *b*, a component of complex III (cytochrome bc_1) of the parasite's electron transport chain. Specifically, DNDI-6174 interacts with the Q_i active site of this mitochondrial enzyme. While cytochrome *b* was identified through chemical genomics as a possible target for new drug discovery efforts aimed at treating Chagas disease (*17*), recent studies have shown that the Q_i site of cytochrome *b* is a promiscuous drug target in *L. donovani* and *T. cruzi* (*9*). Drugs targeting the cytochrome bc_1 complex are in clinical use for treatment of malaria and fungal pneumonia, and cytochrome bc_1 complex was also reported as a promising target for treatment of tuberculosis (*18-20*). To our knowledge there are no other compounds with this MoA that have progressed into development for VL.

DNDI-6174's *in vitro* antiparasitic potency combined with good metabolic stability across species allowed its testing in different *in vivo* models of *Leishmania* infection. Its assessment in both *L. donovani/L. infantum* mouse and hamster models exhibited very high levels of efficacy in all organs at doses as low as 12.5 mg/kg for 5 days or 6.25 mg/kg for 10

days, leading to a reduction of the parasite burden of more than 99% in all organs with a potential to approach sterile cure. Further characterization indicated that despite only moderate solubility, DNDI-6174 showed good permeability and had excellent pharmacokinetic properties (although possible solubility limited absorption and bioavailability at high dose); PK/PD analysis suggested a predicted curative dose in humans ranging between 20-180 mg (qd) or 10-80 mg (bid) for a 10-day dosing period. This predicted human dose regimen, despite its broad dose range, is very favorable and compares well, or is even lower than the standard of care and other NCEs currently in development.

Given its target, potential mitochondrial toxicity of DNDI-6174 was a concern and a specific screening cascade with clear go/no go criteria was developed. In the biochemical assay against the mammalian cytochrome *bc1* complex a therapeutic window above 1000 was determined. In the functional HepG2 cell-based MitoExpress® and Seahorse® assays inhibition of host mitochondrial activity with DNDI-6174 was found to be limited. A reduction in the calcium loading capacity of isolated mitochondria was observed, suggesting the potential for some mito-activity with DNDI-6174, which warrants further consideration. However, the overall pattern across all the endpoints was not consistent with an inhibition of the mammalian cytochrome *bc1* complex. Early receptor and enzyme profiling did not show any relevant flags and a generally good *in vitro* safety profile was observed. Moreover, DNDI-6174 did not show any phototoxicity, genotoxicity, or safety pharmacology flags, and an encouraging therapeutic index following a 14-day exploratory toxicity study in the rat was achieved.

Overall, DNDI-6174 combines encouraging preclinical *in vivo* efficacy with appropriate pharmaceutical properties and an acceptable safety profile fulfilling all DNDi TCP criteria for VL. In addition, it shows potential for CL, as it demonstrated similar levels of *in vitro* activity against species of *Leishmania* responsible for that disease. This makes DNDI-6174 suitable for onward development against all forms of leishmaniasis.

The results are limited by the not yet fully established translation of the *in vivo* models of this disease and consequently, by an understanding of what is the most adequate model for human dose prediction. This is reflected by the large dose range for the predicted human dose due to differences in the efficacy data depending on the species used and the animal model considered (mouse or hamster). Clinical data will provide an indication of the value of each of these models, and which one is more appropriate for use in the future human dose predictions.

Another potential issue is linked to the molecular target of DNDI-6174 itself. Cytochrome *b* is encoded by kinetoplastid DNA, equivalent to mitochondrial DNA in other eukaryotes. Replication of kinetoplast DNA is considered error prone and thus genes encoded by the kinetoplast are associated with a particularly high mutation rate which may lead to a higher resistance potential. However, it should be noted that malaria parasites bearing mutations in cytochrome *b* and resistant to atovaquone are not transmissible by mosquitoes (*21*). The apparent loss of fitness of atovaquone-resistant parasites in the mosquito has been associated with the higher respiratory rate required at this stage of the lifecycle. The failure of these mutated parasites to be transmitted effectively limits the spread of atovaquone resistance in the field. It remains to be seen if a similar fitness cost will be associated with *L. donovani* parasites bearing Q_i site mutations. Regardless, future treatment strategies for VL are focusing on the development of combination therapies to limit the development of resistance to treatment. In that respect, and in the context of development of a combination therapy, more work will be necessary to assess in more detail the potential risk of drug-drug-interactions with DNDI-6174 given its potential time-dependent inhibition of CYP2D6 and CYP3A4/5, and to a lesser degree CYP2C8.

Overall, the data for DNDI-6174 supports its continued development to determine whether this compound can become a much-needed safe oral treatment for patients suffering

from multiple forms of the devastating neglected tropical disease leishmaniasis. It is currently under preclinical development with the goal of starting a clinical phase I trial in 2025.

Materials and methods

Study design

The objective of the research was to generate all data necessary to determine if DNDI-6174 possess properties fulfilling the Target Candidate Profile for visceral leishmaniasis and thereby supporting its selection as a potential preclinical candidate for further development. The research followed the fairly standardized process of drug discovery and development and included experiments, both *in vitro* and *in vivo*, to assess the activity and efficacy of DNDI-6174 against *Leishmania* parasites, the elucidation of its mode of action and identification of its target, the drug metabolism and pharmacokinetic (DMPK) properties of the compound, and its safety profile.

All studies / experimental protocols involving animals were approved by and performed following the respective institutional, national, European and international guidelines (see below for the detailed ethical statements). Study reporting followed the principles of the ARRIVE guidelines 2.0. For efficacy studies, animals fulfilling specific inclusion criteria related to body weight and age, were randomly allocated to experimental units of 5 animals at the experimental start. The sample sizes of five animals per group, approved by the ethical committees, were selected based on a power analysis using G*Power 3.1.9.2 considering the Leishman Donovan Units (LDU) as a parameter for infection and allowing reproducibility and statistical significance between 2 groups (*t-test*) with a minimum power of 95%. For all efficacy studies in mice and hamsters, only female animals were used. While we recognized that this is a potential limitation, male rodents show aggressive behavior over the course of relatively long infection experiments which can impact their hematological / immunological profiles; very often male rodents reach humane endpoints (e.g. heavy scarring, poor overall condition as defined in the Functional Observational Battery) requiring culling of the animals. Approval

from both University of Antwerp (LMPH) and the London School of Hygiene and Tropical Medicine (LSHTM) animal ethical committees has been given for efficacy studies in female animals. Investigators were not blinded to the drug administered (in some cases blinding was impossible due to different modes of administration or visual differences of the test items), nor to downstream analysis of parasite burden (with the exception of the *in vitro* promastigote back transformation assay that was performed by a different investigator than the one conducting the dosing and group allocation). In rare cases, outliers based on Grubbs' test (n=5, α=0.05) were excluded from mean and SD calculations. Details on sample sizes, replicate information and statistical analysis for each experiment are depicted in figures legends and tables footnotes. For pharmacokinetic studies in different species, three animals were used for each dose assessed and data are depicted in figures and tables as mean n=3 +/- SD except where otherwise stated.

For *in vitro* assays, multiple biological replicates were performed. If not otherwise specified in the figure legends or table footnotes, values represent the mean of at least two biological replicates, with each replicate consisting of at least two technical replicates.

Ethical statements

All animal experiments were performed according to institutional ethical guidelines for animal care. More precisely, Sprague Dawley rat and BALB/c mouse PK studies were conducted at Monash University according to protocols reviewed and approved by the Monash Institute of Pharmaceutical Science Animal Ethics Committee and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Sprague Dawley rat and beagle dog PK studies were conducted at WuXi in accordance with institutional and national guidelines at WuXi AppTec (the Institutional Animal Care and Use Committee (IACUC)). Efficacy studies in hamsters and mice at LMPH were carried out in strict accordance with all

mandatory guidelines (EU directives, including the Revised Directive 2010/63/EU on the protection of Animals used for Scientific Purposes that came into force on 01/01/2013, and the declaration of Helsinki in its latest version) and were approved by the ethical committee of the University of Antwerp, Belgium (UA-ECD 2011-74 and 2019-10). The mouse efficacy study at LSHTM was carried out under a UK Home Office project license according to the Animal (Scientific Procedures) Act 1986 and the new European Directive 2010/63/EU. The project license (70/8427) was reviewed by the LSHTM Animal Welfare and Ethical Review Board prior to submission and consequent approval by the UK Home Office. The design of the Wistar rat toxicity study conducted at Charles River Laboratories France Safety Assessment SAS (AAALAC accredited test Facility) was reviewed and approved by the ethical committee of the Test Facility as per the standard project authorization no. 2017072617402851. The study design was in general compliance with the following animal health and welfare guidelines: Guide for the care and use of laboratory animals (2011), Decree n° 2013-118 relating to the protection of animals used in scientific experiments described in the Journal Officiel de la République Française on 01 February 2013, Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Leishmania intramacrophage in vitro assay

Primary peritoneal macrophage (PMM) host cells, in RPMI-1640 medium, supplemented with 2 mM L-glutamine, 16.5 mM $NaHCO_3$, and 5% inactivated fetal calf serum, were infected at a ratio of 1:5 with *L. infantum* MHOM/MA(BE)/67/ITMAP263 spleen-derived amastigotes isolated from heavily infected donor hamsters or at a ratio of 1:15 with late stationary-phase promastigotes of the different VL and CL strains (Table **1**). A drug incubation period of 96 h was applied, and cells were stained with Giemsa for microscopic evaluation of cellular

amastigote burdens. Percentage reduction compared to the burden in the infected non-treated control wells was used as a measure for drug activity.

Alternatively, differentiated macrophage host cells derived from THP-1 (human leukemia monocytes) were infected at a multiplicity of infection of 1:2.5 (ratio of host cells to parasites) with *L. donovani* MHOM/IN/80/DD8 culture-maintained promastigotes. *L. donovani* MHOM/IN/80/DD8 (ATCC 50212) promastigote parasites and THP-1 (ATCC TIB202) cells were maintained as previously described (*22*). A drug incubation period of 96 h was applied, plates fixed with 4% paraformaldehyde and stained with SYBR® green and CellMask Deep Red™ (ThermoFisher Scientific). Images were acquired on an Opera high-content imaging system (PerkinElmer). Healthy host (THP-1) cells and intracellular amastigotes were identified using CellMask Deep Red cytoplasmic and SYBR green nuclear area and intensities, with intracellular parasites identified based on spot detection algorithms (size and intensity measurements used to define parasite nucleus) to determine the number of parasites present within THP-1 host cells. An infected cell was defined as a host cell containing >3 parasites within the cytoplasm boundary. Compound activity was determined based on the number of infected cells normalized to the positive (10 μM DNDI-1044) and negative (0.4% DMSO) controls. Non-linear sigmoidal dose-response curves with no constraints were plotted, and IC_{50} was calculated. The IC_{50}s were calculated from two independent experiments.

In vivo efficacy studies in mice and hamsters

All animal experiments were performed according to the respective institutional ethical guidelines for animal care and in compliance with the national and international animal health and welfare guidelines (see Ethical statement).

Mouse infection model (VL). 8−10-week-old and 15-20 grams body weight (inclusion criteria) female BALB/c mice were purchased from Janvier, France, and kept in quarantine for at least

5 days before starting the experiments. Animals were maintained under specific pathogen-free conditions in individually ventilated cages and experienced a 12-hour light/dark cycle. Food for laboratory rodents and drinking water were available ad libitum. The animals were randomly allocated to experimental units of 5 animals based on live body weight at experimental start and infected with 2 x 10^7 *L. donovani* MHOM/ET/67/L82 (synonym HU3) or *L. infantum* MHOM/MA67/ITMAP263 amastigotes on day 0 (intravenous administration in the lateral tail vein of BALB/c mice; infection inoculum volume of 100 µl). On day 6 post-infection, treatment with the designated test item was initiated by oral gavage once a day for 5 or 10 days. DNDI-6174 was formulated in 0.5% (w/v) hydroxypropylmethylcellulose (HPMC), 0.4% (v/v) benzyl alcohol and 0.5% (v/v) Tween 80 in water, miltefosine was prepared in distilled water, while AmBisome for IV administration was formulated in 5% dextrose. Due to the different visual appearance of vehicle compared to formulated compound and reference drugs, as well as the route of administration, the investigator could not be blinded during the administration process. Five days following the end of treatment, all mice were sacrificed, and parasite burden was determined from smears of liver sections. Efficacy was expressed as the mean percentage load reduction compared to untreated (vehicle-only) control animals. Reference drugs miltefosine and/or AmBisome were used as positive controls and comparators in each study. The total number of animals for each study arm is shown in the relevant Tables (supplementary material). Animals were observed daily for the occurrence/presence of clinical or adverse effects during the course of the experiment. In case of very severe clinical signs or unexpected toxicity, animals were euthanized for animal welfare reasons. All animals were weighed twice weekly during the experiment to monitor the general health status (severity of infection, toxicity of medication).

<u>*Hamster infection model (VL).*</u> Female golden hamsters of 75 to 80 g body weight (inclusion criteria) were purchased from a commercial source (Janvier, France) and kept in quarantine for

at least 5 days before starting the experiment. Animals were maintained under specific pathogen-free conditions in individually ventilated cages and experienced a 12-hour light/dark cycle. Food for laboratory rodents and drinking water were available ad libitum. The animals were randomly allocated to experimental units of 5 animals each, based on body weight at the start of the experiment and were infected through intracardiac administration of $2x10^7$ *L. infantum* amastigotes obtained from the spleen of a heavily infected donor hamster (inoculum of 100 µl PBS). DNDI-6174 (formulated in 0.5% (w/v) HPMC, 0.4% (v/v) benzyl alcohol and 0.5% (v/v) Tween 80 in water), miltefosine (formulated in distilled water) as reference control or vehicle (0.5% (w/v) HPMC, 0.4% (v/v) benzyl alcohol and 0.5% (v/v) Tween 80 in water) were administrated orally from day 21 post-infection. Due to the different visual appearance of vehicle compared to formulated compound and reference drug, the investigator could not be blinded during the administration process. After 5-day treatment period, followed by a 10-day wash out period, animals were sacrificed, and parasite burden assessed in the three target organs (liver, spleen, and bone-marrow). For this assessment, organs of individual animals were weighed, except for bone-marrow thus providing a semi-quantitative evaluation only. Impression smears were Giemsa-stained for microscopic evaluation of amastigote burden, expressed as LDU (= mean number of amastigotes/cell × organ weight in mg) and the results were expressed as a percentage reduction compared to the burden in the control group (vehicle as placebo). For evaluating the presence of viable residual burden after treatment, a promastigote back-transformation assay was conducted. This consisted of the incubation at ambient temperature of aseptically collected pieces of spleen or liver tissue in 1 mL of promastigote back-transformation medium in 24-well plates. For the bone-marrow, resected femurs were flushed with 1 mL medium. The medium consists of HOMEM pH 6.0 prepared as described elsewhere (*8*) with 10% fetal bovine serum (Gibco), 2.5% penicillin/streptomycin and 1% gentamicin (Merck). A qualitative assessment of the presence of promastigotes was

made after 3 and 5 days of incubation and a score was attributed (+, ++ or +++) based on parasite density in the positive wells. Animals were observed daily for the occurrence/presence of clinical or adverse effects during the course of the experiment. In the case of very severe clinical signs or unexpected toxicity, animals were euthanized for animal welfare reasons. All animals were weighed twice weekly during the experiment to monitor the general health status (severity of infection, toxicity of medication).

Cell lines and culture conditions (mode of action studies)

The clonal *L. donovani* cell line LdBOB (derived from MHOM/SD/62/1S-CL2D) was grown as promastigotes at 28°C and as axenic amastigotes at 37°C, as described previously (*23*).

Drug sensitivity assays (mode of action studies)

To examine the effects of test compounds on growth in 96-well plates, promastigote or axenic amastigote cultures seeded at 5×10^4 parasites mL^{-1} were incubated in the presence of 2-fold serial dilutions of test compounds for 72 h. Following incubation, 50 µM resazurin was added to each well and fluorescence (excitation of 528 nm and emission of 590 nm) measured after a further 3 h incubation. Data were processed using GRAFIT (Erithacus software) and fitted to a 2-parameter equation, where the data are corrected for background fluorescence, to obtain the effective concentration inhibiting growth by 50% (EC_{50}):

$$y = \frac{100}{1 + \left(\frac{[I]}{EC_{50}} \right)^m} \qquad [1]$$

In this equation $[I]$ represents inhibitor concentration and m is the slope factor. Experiments were repeated at least two times and the data is presented as the mean plus standard deviation.

Generation of compound-resistant parasites

Compound-resistant lines were generated by subculturing clones of WT *L. donovani* in the continuous presence of DNDI-6174. Starting at sublethal concentrations, drug concentrations in 5 independent cultures were increased in a stepwise manner, usually by 2-fold. When parasites were able to survive and grow in 1 µM DNDI-6174, the resulting lines were cloned by limiting dilution in the presence of compound. Five clones (Res 1–5) were selected for further biological study.

Whole genome sequencing

Genomic DNA was collected from wild type and resistant line *Leishmania donovani* promastigotes and sequenced by the Beijing Genomics Institute (BGI). Sequence reads were aligned to the *L. donovani* BPK282A1 genome (v39, tritrypDB) with maxi-circle (CP022652.1, NCBI) as described previously (*9*). Median read counts of the wild type and resistant lines were used to normalise copy number. The associated data sets have been deposited with the European Nucleotide Archive under the following accession number: PRJEB45584.

Complex III assays

Measurement of complex III activity and inhibition were performed as described previously (*9*).

In vitro metabolism

The *in vitro* intrinsic clearance of DNDI-6174 was determined in human, dog, rat and mouse liver microsomes (Sekisui XenoTech, LLC, Kansas City, KS) at a substrate concentration of 0.5 µM and a protein concentration of 1 mg/mL. Compounds were spiked into microsomal matrix prepared in 0.1 M pH 7.4 phosphate buffer containing magnesium chloride (final concentration of 3.3 mM), equilibrated for 5-10 min at 37°C. The reaction was initiated with

the addition of freshly prepared solution of NADPH (final 1.3 mM). Control samples without co-factor were included for comparison. Aliquots of the reaction mixtures were taken periodically over 60 min and quenched with acetonitrile containing internal standards (metolazone and diazepam). Quenched samples were maintained on ice for approximately 20-30 min, centrifuged (for 5 min) and the supernatant was analyzed by LC-MS.

The *in vitro* intrinsic clearance of DNDI-6174 was also assessed in human, dog, rat and mouse cryopreserved hepatocytes (Sekisui XenoTech) suspended in Krebs-Henseleit buffer at 0.5 µM substrate and a cell density of $1x10^6$ viable cells/mL. Cell viability was determined using trypan blue exclusion. The incubation was performed on a plate shaker (Heidolph Titrimax 100, 900 rpm) placed in a humidified incubator (37°C, 7.5% CO_2) for 240 min. The reaction was initiated with the addition of compound with samples taken periodically and quenched with the addition of acetonitrile containing internal standards (metolazone and diazepam). Quenched samples were maintained on ice for approximately 20-30 min, centrifuged (for 5 min) and the supernatant analyzed by LC-MS.

The LC-MS conditions for both microsomal and hepatocyte samples consisted of a Waters Xevo G2-S QToF coupled to a Waters Acquity UPLC, an Ascentis Express C8 column (50 x 2.1 mm, 2.7 µm) and a mobile phase consisting of water and acetonitrile, each containing 0.05% formic acid and delivered under gradient conditions over 6 min. The injection volume was 3 µL and the flow rate was 0.4 mL/min. MS was conducted in positive mode electrospray ionization under MS^E acquisition mode to allow simultaneous acquisition of MS spectra at low and high collision energies.

Metabolite detection was conducted using Waters UNIFI software and metabolites were identified using a combination of accurate mass, MS/MS (CID) spectral analysis and comparison of retention times and CID spectra to those of authentic metabolite standards where available.

In vitro intrinsic clearance (CL_{int}, µL/min/mg or µL/min/10^6 cells) values were calculated from the apparent first order degradation rate constant divided by the microsome concentration (mg/mL) or the hepatocyte cell number (10^6 viable cells/mL). *In vitro* CL_{int} values were then divided by the fraction unbound (f_u) in the *in vitro* test system (to give $CL_{int,u}$) and scaled to *in vivo* values (mL/min/kg) using published physiological scaling factors (*24*). Blood clearance in each species was then calculated using the well-stirred model of hepatic extraction (equation 2) where Q is the hepatic blood flow and $f_{u,blood}$ is calculated from $f_{u,plasma}$/B:P where B:P is the blood to plasma ratio:

$$Predicted\ CL_{blood} = \frac{Q\ x\ f_{u,blood}\ x\ CL_{int,u}}{Q + f_{u,blood}\ x\ CL_{int,u}} \qquad [2]$$

Blood clearance values were converted to plasma clearance by multiplying by the B:P.

In vivo pharmacokinetic properties

Single dose intravenous (IV) PK studies in male Sprague Dawley rats and IV and oral PK studies in female BALB/c mice were conducted at Monash University. DNDI-6174 was administered by IV infusion (n=3 rats) over 10 min via a cannula implanted in the jugular vein on the day prior to dosing at a dose of 3 mg/kg prepared in 0.9% saline containing 5% DMSO and 2% Solutol HS-15 (1 mL dose volume). Blood was sampled via a cannula implanted in the carotid artery (also on the day prior to dosing) at pre-determined time points up to 24 h post-dose. Samples were transferred to vials containing heparin as an anticoagulant, gently mixed and centrifuged, and plasma separated for subsequent quantitative analysis using LC-MS. For mice, DNDI-6174 was administered by IV bolus injection into the lateral tail vein (2 mL/kg) at a dose of 2 mg/kg using the same IV formulation vehicle as for rats. A dose of 25 mg/kg

prepared in an aqueous suspension vehicle containing 0.5% (w/v) HPMC, 0.5% (v/v) benzyl alcohol and 0.4% (v/v) Tween 80 (0.2 mL per mouse) was also dosed via oral gavage. Mouse blood was sampled via submandibular bleed up to 24 h post-dose (n=2 mice per time point, n=3 samples per mouse) into vials containing heparin and plasma was separated for quantitative analysis as described above.

Rat, mouse, hamster and dog pharmacokinetic studies

Additional PK studies were conducted by WuXi AppTec Co, Ltd (Shanghai, China) according to protocols reviewed and approved by DNDi. Studies were conducted in male Sprague Dawley rats (IV dose of 3 mg/kg and single oral doses of 10, 50, 100 and 300 mg/kg), female BALB/c mice (twice daily (at 6 and 24 h) oral doses of 3.13, 6.25 and 12.5 mg/kg for 5 days), golden Syrian hamsters (single oral dose of 50 mg/kg and once daily oral doses of 6.25, 12.5 and 25 mg/kg for 5 days) and in beagle dogs (IV dose of 1 mg/kg and single oral doses of 5 and 30 mg/kg). IV and oral formulations for rats, mice and hamsters were the same as described above. For dogs, the IV dose was administered in saline containing 2% DMSO and 3% Solutol HS-15 and the oral formulation was as described above. Blood was sampled up to 48 h post-dose into vials containing potassium EDTA as an anticoagulant, proteins were precipitated with acetonitrile and plasma was separated and assayed by LC-MS. PK data analysis methods are described in the Supporting Information.

Bioanalytical methods

At Monash University, plasma samples were assayed by LC-MS/MS following protein precipitation and quantitated against calibration standards prepared in blank matrix. Briefly, proteins were precipitated by the addition of acetonitrile (3:1 volume ratio) containing diazepam as an internal standard after which samples were centrifuged and the supernatant injected onto the LC-MS system. Urine samples were treated with an equal volume of

acetonitrile and assayed against calibration standards prepared in 50% acetonitrile/water with diazepam as the internal standard. The LC-MS system consisted of a Waters Acquity UPLC coupled to either a Waters Xevo TQS Micro or Waters Xevo TQ mass spectrometer. Chromatography was conducted using a Supelco Ascentis Express RP Amide column (50 x 2.1 mm, 2.7 µm) maintained at 40°C and a mobile phase consisting of phase A (water containing 0.05% v/v formic acid) and B (acetonitrile containing 0.05% v/v formic acid) and mixed under gradient elution conditions (4 min cycle, 0.4 mL/min flow rate). MS conditions included ESI positive mode with multiple reaction monitoring using cone and CID voltages of 50 V and 35 V, respectively. The elution of DNDI-6174 was monitored using a transition (m/z) of 308.17 > 280.17. The calibration range was from 1 to 10,000 ng/mL and the lower limit of quantitation was 1 ng/mL. Precision (% relative standard deviation) and accuracy (% bias) were <5% and within ± 15%, respectively.

For studies conducted at WuXi, plasma samples were assayed using a similar procedure as described above with the exception of the column (Acquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm) maintained at 60°C) and mobile phase (phase A: 95% water (containing 0.1% formic acid and 2 mM ammonium formate)/5% acetonitrile; phase B: 95% acetonitrile (containing 0.1% formic acid and 2 mM ammonium formate)/5% water) mixed under gradient conditions over a 4 min cycle with a flow rate of 0.6 mL/min.

AP-preDICT simulation

ApPredict is a simulation engine, a bolt-on extension to the software package Chaste, to perform simulations of drug-induced changes to the cardiac action potential (https://chaste.cs.ox.ac.uk/trac/wiki/ApPredict). This *in silico* model of human and rabbit cardiac myocyte allows prediction of the action potential response to ion channel modulation.

Statistical analysis

All data are shown as mean +/- SD. Student's *t* tests were used to compare two groups (e.g. dosing regimen vs vehicle). One-way analysis of variance (ANOVA) testing for significance at $\alpha=0.05$ was used to detect differences between more than two groups. Statistical analysis were performed using GraphPad Prism 6.0 software. The sample size for pharmacodynamic studies in animal experiments was based on a power analysis using G*Power 3.1.9.2 considering LDU (Leishman Donovan Units) as a parameter for infection (*t*-test, $\alpha=0.05$). Potential outliers based on Grubbs' test ($n=5$, $\alpha=0.05$) were excluded from the means and SD calculations.

List of supplementary materials

Material and methods
Supplementary information on Tables

Fig. S1 Chromosomal CNV relative to wild-type in DNDI-6174-resistant clones.

Fig. S2 Binding mode of DNDI-6174 in the Q_i sites of mutated versions of cytochrome *b* (A-E). Ser35Asn and Ser206Asn mutations identified in cell line Res 1 are reported in magenta and green (A). Mutation Asp231Glu identified in Res 2 is represented in orange (B). The Ser207Pro mutation identified in Res 3 is in light blue (C). Gly31Ala (Res 4) (D) and Ser206Asn (Res 5) (E) are reported in wheat and green, respectively.

Fig. S3 (A) The Ligand Root Mean Square Fluctuation (RMSF) for each atom of DNDI-6174 in cytochrome *b* (wild-type) measuring the changes in the position of ligand atoms during the 100 ns MD simulation (atom number as reported in the chemical representation on the right). These studies indicate that benzodioxole moiety of DNDI-6174 is the portion of the molecule with the highest flexibility during binding. (B) A schematic of detailed ligand atom interactions with the amino acid residues of cytochrome *b*. (C) Root Mean Square Deviation (RMSD) plot of DNDI-6174 (right Y-axis – magenta curve) and wild-type cytochrome *b* (left Y-axis – blue curve). The plot indicates the stability of the protein and the ligand during the simulation. The overall stability of the docking binding pose is highlighted by the protein and ligand RMSD over this simulation. (D) Protein-Ligand Contacts plot. The possible interactions are categorized into four types: hydrogen bonds, hydrophobic, ionic and water bridges. The stacked bar charts are normalized over the course of the trajectory.

Fig. S4 (A) The Ligand Root Mean Square Fluctuation (RMSF) of DNDI-6174 during the 100 ns MD simulation in the mutated cytochrome *b* (Ser35Asn/Ser206Asn) from Res 1. Ligand flexibility in this mutated enzyme increased considerably, even for the pyrrolopyrimidine scaffold that was particularly tightly bound in the wild-type enzyme. RMSF is reported by atom number as reported in the chemical representation on the right. (B) A schematic of detailed ligand atom interactions with the protein residues. (C) Root Mean Square Deviation (RMSD) plot of DNDI-6174 and cytochrome b. (D) Protein-Ligand Contacts plot. The possible interactions are categorized into four types: hydrogen bonds, hydrophobic, ionic and water bridges. The stacked bar charts are normalized over the course of the trajectory. The mutation of 206 from Ser to Asn dislodges the ligand from its binding site, breaking a critical H-bond with Asp231 and thus contributing to the decrease in ligand binding stability compared to that seen with the wild type enzyme. The Ser35Asn mutation that accompanies Ser206Asn is not located in the binding site, and its role in drug resistance seems to be the result of an indirect interaction between the residue and the ligand.

Fig. S5 (A) RMSF of DNDI-6174 during the 100 ns MD simulation in the mutated cytochrome *b* from Res 5 (Ser206Asn). RMSF is reported by atom number as reported in the chemical representation on the right. (B) A schematic of detailed ligand atom interactions with the protein residues. (C) RMSD plot of DNDI-6174 and cytochrome b. (D) Protein-ligand contacts plot. The possible interactions are categorized into four types: hydrogen bonds, hydrophobic, ionic and water

bridges. The stacked bar charts are normalized over the course of the trajectory. See Fig. S4 legend for details of the impact of this mutation on ligand stability.

Fig. S6 (A) RMSF of DNDI-6174 during the 100 ns MD simulation in the mutated cytochrome b from Res 2 (Asp231Glu). RMSF is reported by atom number as reported in the chemical representation on the right. (B) A schematic of detailed ligand atom interactions with the protein residues. (C) RMSD plot of DNDI-6174 and cytochrome b. (D) Protein-ligand contacts plot. The possible interactions are categorized into four types: hydrogen bonds, hydrophobic, ionic and water bridges. The stacked bar charts are normalized over the course of the trajectory. Our MD analysis strongly suggests that the longer and more flexible Glu231 side chain bends away from the ligand. This new conformation of the Glu231 side chain results in a complete loss of interactions with the ligand, reflected in a 164-fold reduction in DNDI-6174 potency compared to the wild-type (Table **2**).

Fig. S7 (A) RMSF of DNDI-6174 during the 100 ns MD simulation in the mutated cytochrome *b* from Res 3 (Ser207Pro). RMSF is reported by atom number as reported in the chemical representation on the right. (B) A schematic of detailed ligand atom interactions with the protein residues. (C) RMSD plot of DNDI-6174 and cytochrome b. (D) Protein-ligand contacts plot. The possible interactions are categorized into four types: hydrogen bonds, hydrophobic, ionic and water bridges. The stacked bar charts are normalized over the course of the trajectory. The Ser207Pro mutation impacts ligand binding by changing the morphology of the binding site. The mutation causes the rearrangement of secondary structure elements, ultimately disrupting the key H-bonds interactions between the Asp231 side chain and the 2-amino group of DNDI-6174.

Fig. S8 RMSF of DNDI-6174 during the 100 ns MD simulation in the mutated cytochrome b from Res 4 (Gly31Ala). RMSF is reported by atom number as reported in the chemical representation on the right. (B) A schematic of detailed ligand atom interactions with the protein residues. (C) RMSD plot of DNDI-6174 and cytochrome b. (D) Protein-ligand contacts plot. The possible interactions are categorized into four types: hydrogen bonds, hydrophobic, ionic and water bridges. The stacked bar charts are normalized over the course of the trajectory. Our data indicates that the resistance observed in the Gly31Ala mutated results in steric clashes. The methyl group of the Ala side chain displaces the conserved water molecule bridging the interaction between the ligand and Phe34 and disrupts the interaction with Asp231 by clashes with the NH_2 of the 2-aminopyrrolopyrimidine core.

Fig. S9 Degradation profiles for DNDI-6174 incubated with human liver microsomes in the absence and presence of inhibitors specific for individual cytochrome P450 (CYP) isoforms. There were no significant differences in the degradation slopes in the absence and presence of inhibitor for any isoform. Statistical comparison of the degradation slopes was conducted using an extra sum-of-squares F-test testing for significance at $\alpha = 0.05$ (GraphPad Prism ver 9.3.1).

Fig. S10 Metabolite formation profiles for DNDI-6174 metabolites formed following incubation with human liver microsomes in the absence and presence of inhibitors specific for individual cytochrome P450 (CYP) isoforms.

Fig. S11 Simulated and **e**xperimental plasma PK data following (A) twice-daily oral administration (doses given at 6 and 24 h) for 5 days in mice and (B) once-daily

oral administration for 5 days in hamsters. Note that at the highest dose of 25 (mice) and 50 (hamsters) mg/kg, only a single dose was administered. Symbols represent the measured data (mean ± SD, n=3) and lines represent the simulated data based on the best-fit using a one compartment body model.

Fig. S12 Plasma AUC_{24} at steady state ($AUC_{24\ ss}$) and C_{max} as a function of dose for hamsters (qd dosing for 5 days, n=3) and mice (bid dosing at 8 and 24 h for 5 days, n=3) based on compartmental fits of the experimental concentration versus time data (black symbols ± SD). Experimental data on day 1 and day 5 of dosing are shown with blue and red symbols, respectively. Mouse C_{max} data on day 1 were taken after the first dose and on day 5, data were taken after the second dose.

Fig. S13 Simulated repeat dose plasma profiles for DNDI-6174 in mice and hamsters following once or twice-daily oral administration (8 and 24 h) for 5 days as used in the efficacy studies.

Fig. S14 Simulated human plasma concentration vs time profiles to achieve a cumulative plasma AUC of between 60 and 460 µg.h/mL. Profiles were simulated using GastroPlus and the parameters shown in Table **S16**.

Fig. S15 DNDI-6174 was tested in hIPSc-CM (cardiomyocytes) MEA (multielectrode) assay at concentrations of 0.37, 1.11, 3.33, 10 and 30 µM. Concentrations were selected based on estimated free C_{max} at efficacious exposure and potency against cardiac ion channel data: Qpatch hERG IC_{50} = 31.6 µM; Qube NaV1.5 IC_{50} > 50 µM; Qube CaV1.2 IC_{50} > 100 µM (43% inhibition). DNDI-6174 produced no notable change in the field potential duration (FPD), the spike amplitude and the beat period throughout predicting no significant QT prolongation and no notable change in cardiac conduction.

Table S1 *In vivo* efficacy of DNDI-6174 and positive controls (AmBisome or miltefosine) in (A) the mouse model and (B) the hamster model. Data for organ burden are expressed as a % of the vehicle control in the same experiment (mean n=5 ± SD).

Table S2 Results for the promastigote transformation assay where organs from hamsters treated with DNDI-6174, miltefosine or vehicle control were cultured *in vitro* and monitored for the emergence of viable parasites (promastigotes). Results represent an arbitrary parasite score attributed (+, ++ or +++) based on visual inspection of parasite density in the positive wells. A score of "-" is attributed in the absence of parasite. Scores are reported individually, 7 days post autopsy, from the three target organs (liver, spleen and bone-marrow).

Table S3 Summary of read counts and coverage for whole genome sequencing of DNDI-6174-resistant clones.

Table S4 Summary of non-synonymous SNPs identified in whole genome sequencing of DNDI-6174-resistant parasites.

Table S5 Physicochemical, permeability and binding properties for DNDI-6174

Table S6 *In vitro* intrinsic clearance (CL_{int}, ± standard error of estimate) of DNDI-6174 following incubation with liver microsomes (2 independent experiments) and cryopreserved hepatocytes (single experiment) and predicted *in vivo* plasma clearance. The measured *in vivo* plasma clearance from Table **S7** is shown for reference.

Table S7 Intravenous and oral plasma pharmacokinetic properties of DNDI-6174 in mice, rats, and dogs following single dose administration.

Table S8 Oral plasma exposure of DNDI-6174 in rats following a single dose (mean n=3 ± S.D.).

Table S9 Oral plasma exposure of DNDI-6174 in dogs following a single dose (mean n=3 ± S.D.)

Table S10 Oral plasma exposure of DNDI-6174 in mice following twice daily (at 6 and 24 h) dosing for 5 days (mean n=3 ± S.D.).

Table S11 Oral plasma exposure of DNDI-6174 in hamsters following once daily dosing for 5 days (mean of n=3 ± S.D.).

Table S12 Summary of fitted plasma compartmental parameters for DNDI-6174 following single oral dosing to mice and hamsters.

Table S13 Pharmacodynamic data (liver burden, mean n=5 ± SD) for mice infected with *L. infantum* or *L. donovani* and treated with DNDI-6174. For the liver burden data, the SD is shown in parentheses and for the plasma PK parameters, the unbound values are shown in parentheses. Pharmacokinetic parameters are from the fitted analysis as described above.

Table S14 Pharmacodynamic data (liver burden, mean n=5 ± SD, Table **S1**) for DNDI-6174 in hamsters infected with *L. infantum*. Pharmacokinetic parameters are from the fitted analysis as described above. Unbound PK parameters are shown in parentheses.

Table S15 Best fit parameters for the data shown in Figure 6 obtained using a 4-parameter logistic function. Values in parentheses represent the standard errors of the fitted parameters.

Table S16 Input parameters for GastroPlus simulations.

Table S17 Early *in silico* and *in vitro* cardiotoxicity assessment of DNDI-6174.

Table S18 Complete *in vitro* cytotoxicity profiling of DNDI-6174.

Table S19 DNDI-6174 profile in a panel mammalian receptors, enzymes and ion channels.

Table S20 Assessment of human complex III activity and mitochondrial toxicity.

Table S21 Percentage inhibition of human bc_1 complex (Complex III) in presence of DNDI-6174 at various concentrations.

Table S22 Cytochrome P450 inhibition by DNDI-6174.

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Author contributions: SB, ECh, and MK conceived the idea for the project. SB, SH, GCa, LM, BZ, VA, MK, KJB, JH, MA, RI, DS, SAC, SC, SW, RJW, FZ, JJL, GB, M-MM, and PCC developed or designed the methodology and assays / models used in these studies. KB, JH, MA, and RI performed the chemical synthesis, KK, ECr, GCh, RP, GL DMS, and KLW performed the *in vitro* and *in vivo* ADME and PK analysis, SAC conducted the human PK and dose predictions, P-BF, NVP, AM, SH, and VY, performed the *in vivo* efficacy studies in mice and hamsters, BZ performed the *in vitro* intramacrophagic *Leishmania* assays, SC generated the compound-resistant parasites and performed the drug sensitivity assays (mode of action studies), RJW and FZ, performed the molecular modelling, GB, DS, JD, and IC performed the complex III inhibition assays, the mitochondrial function assessments (MitoXpress and Seahorse® assays) and calcium loading capacity (CLC) assessment in HEK293F-derived mitochondria. SB, SW and SAC performed the visualization of the data. SB, ECh, SAC, SW,

GCa, LM, SG and MK were responsible for the administration of the project. ECh, MK, SAC, SW, GCa, LM, VA, FZ, SG, MM and JJL supervised the studies. ECh, SAC and SW were involved in funding acquisition. SB, ECh, SAC, MK, SW and FZ wrote the original draft. SB, ECh, SAC, SW, MK, SH, GCa, LM, VA, VY, SG, and JJL reviewed and edited the original draft. All authors read, revised and authorized the manuscript before submission.

Competing interests: SG, MM, JD, DS, GB, JJL, PCC, IC, M-MM are employees of GSK. SG, MM, DS, GB, JJL, PCC, M-MM hold GSK shares. MK, KB, JH, ECh, are named inventors on the patent application EP4092030 A1 - "Pyrrolopirimidine derivatives and their use in the treatment of Leishmaniasis" describing compounds included in the manuscript.

Data and materials availability: All data associated with this study are available in the main text or the Supplementary Materials. Requests for DNDI-6174 should be addressed to SB and will be handled through a material transfer agreement with DNDi. Primary data from figures are in data file S1. All other data have been deposited on Dryad and are available through the following links: molecular modelling data (corresponding to Fig. S3 to Fig. S8: https://doi.org/10.5061/dryad.44j0zpcm8 ; Exploratory toxicology data / reports: https://doi.org/10.5061/dryad.8gtht76vz .

Figure Captions

Fig. 1 Structures of the initial hit TCMDC-143610 and DNDI-6174.

Fig. 2 DNDI-6174 targets the Q_i site of *L. donovani* cytochrome *b*. (A) Representative EC_{50} curves for DNDI-6174 against WT promastigotes (open circles) and DDD01716002 Res 1, 2 and 3 (closed circles, squares and triangles, respectively). The curves are the non-linear fits of data using a two-parameter EC_{50} equation provided by GraFit. EC_{50} values of 22 ± 2, 1276 ± 2, 829 ± 6 and 84 ± 3 nM were determined for WT and DDD01716002 Res 1, 2 and 3 lines, respectively. Composite potency data for these cell lines is shown in Table **2**. (B) Graphical representation of the generation of DNDI-6174-resistant *L. donovani* promastigote lines. Each passage of cells in culture is shown, with clones 1- 5 indicated as open circles, closed circles, open squares, closed squares and open triangles, respectively. Note that some points are overlapping and therefore obscured from view. (C) Secondary structure model of the *L. donovani* cytochrome *b* based on the *Saccharomyces cerevisiae* enzyme (*10*). Amino acids in cytochrome *b* that were mutated in DNDI-6174 resistant lines are indicated by light blue circles. All mutations cluster in close proximity to the Q_i site of cytochrome *b*.

Fig. 3 Binding mode of DNDI-6174 in the Q_i site of *L. donovani* cytochrome *b* evaluated through molecular docking. Docking mode for DNDI-6174 in wild-type cytochrome *b*. Key binding site residues are highlighted as blue-white sticks, with the heme (b_H) cofactor and a conserved water molecule also indicated. The hydrogen bond interactions are indicated as yellow dashed lines and the π-π stackings in cyan.

Fig. 4 A. Proposed hepatic metabolism pathways for DNDI-6174 (DNDI0003366174). Data generated *in vitro* following incubation with liver microsomes or cryopreserved hepatocytes. B. Possible metabolites that were synthetized and confirmed not to be metabolite M+16(II) by comparison of retention times and collision-induced dissociation (CID) fragmentation patterns.

Fig. 5 Intravenous (A) and oral (B) plasma concentration versus time profiles for DNDI-6174 following single dosing to mice, rats, hamsters and dogs. IV dosing to hamsters was not conducted. To facilitate species comparisons, the IV data have been normalized to a dose of 1 mg/kg and the oral to a dose of 10 mg/kg, assuming linear kinetics across the dose range. Actual data at the administered doses are shown in Table **S7**.

Fig. 6 Reduction in liver burden relative to control as a function of cumulative DNDI-6174 unbound AUC in *L. infantum*-infected mice and hamsters. AUC_{95} reflects the cumulative AUC required to achieve greater than 95% reduction in liver parasite burden.

Tables

Table 1. *In vitro* activity (EC_{50} and EC_{90}) of DNDI-6174 against the amastigote stage of different *Leishmania* species/strains.

	Species and strains	EC_{50} (nM)	EC_{90} (nM)
VL strains	*L. donovani* MHOM/IN/80/DD8	170	490
	L. donovani MHOM/ET/67/HU3	90	2000
	L. donovani MHOM/NP/03/BPK275/0-cl18	40	120
	L. donovani MHOM/ NP/2003/ BPK282/0-cl4	50	140
	L. donovani MHOM/ET/2007/LLM-1600	80	280
	L. infantum MHOM/MA/67/ITMAP263	160	440
	L. infantum MHOM/BR/2007/WC (L3015)	70	180
	L. infantum MHOM/ES/2016/LLM-2346	210	1520
	L. infantum MHOM/FR/96/LEM3323	50	180
	L. infantum MHOM/FR/96/LEM3323 Cl4 MIL-R *	70	220
	L. infantum MHOM/FR/96/LEM3323 Cl4 PM-R *	80	170
CL Strains	*L. major* MHOM/SA/85/JISH118	740	11400
	L. major MHOM/IL/81/Friedlin	90	900
	L. guyanensis MHOM/PE/02/PER 054/0	12	39
	L. tropica MHOM/AF/2015/HTD7	360	2180
	L. panamensis MHOM/PA/71/LS94	40	85

EC_{50} values represent the weighted mean of at least three biological replicates with each biological replicate comprised of two technical replicates. * see additional information in the supplementary section.

Table 2. Potency of DNDI-6174 against wild-type (WT) *L. donovani* (LdBOB derived from MHOM/SD/62/1S-CL2D (*23*), DDD01716002- and DNDI-6174-resistant *L. donovani* promastigote cultures.

Cell lines	EC_{50} values, nM	Fold-change versus WT	Cytochrome *b* mutation
Wild-type	24 ± 1	-	-
DDD01716002 Res 1	779 ± 98	32	S207P
DDD01716002 Res 2	829 ± 63	34	G31A
DDD01716002 Res 3	84 ± 3	3.5	F227I
DNDI-6174 Res 1	431 ± 16	18	S35N/S206N
DNDI-6174 Res 2	3946 ± 228	164	D231E
DNDI-6174 Res 3	1500 ± 91	62.5	S207P
DNDI-6174 Res 4	2699 ± 145	112	G31A
DNDI-6174 Res 5	157 ± 5	6	S206N

EC_{50} values represent the weighted mean ± standard deviation of at least three biological replicates with each biological replicate comprised of two technical replicates.

Table 3 - DNDI-6174 potency against developmental stages of *L. donovani* and complex III assays.

Developmental stage	EC_{50} value, nM	Complex III, IC_{50} values, nM
Promastigote	24 ± 1	8 ± 1.7
Axenic amastigote	75 ± 2	2 ± 0.5

EC_{50} values represent the weighted mean ± standard deviation of three biological replicates ($n \geq 3$) with each biological replicate comprised of two technical replicates. IC_{50} values represent the weighted mean ± standard deviation of at least three biological replicates ($n \geq 3$).