Title/Running Title: Pharmacokinetics and pharmacodynamics of the nitroimidazole DNDI-0690 in mouse models of cutaneous leishmaniasis

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Abstract:
The nitroimidazole DNDI-0690 is a clinical drug candidate for visceral leishmaniasis (VL) that also shows potent in vitro and in vivo activity against cutaneous leishmaniasis (CL). To support further development of this compound into a patient-friendly oral or topical formulation for CL, we investigated the free drug exposure at the dermal site of infection and subsequent
elimination of the causative *Leishmania* pathogen. This study evaluates the pharmacokinetics (PK) and pharmacodynamics (PD) of DNDI-0690 in mouse models of CL. Skin microdialysis and Franz diffusion cell permeation studies revealed that DNDI-0690 permeated poorly (< 1 %) into the skin lesion upon topical drug application (0.063 % W/V, 30 µl). In contrast, a single oral dose of 50 mg/kg resulted in the rapid and near-complete distribution of protein-unbound DNDI-0690 from the plasma into the infected dermis (fAUC₀⁻⁶h, tissue / fAUC₀⁻⁶h blood > 80 %). Based on *in vivo* bioluminescence imaging, two doses of 50 mg/kg DNDI-0690 were sufficient to reduce *L. mexicana* parasite load by 100-fold, while 6 such doses were needed to achieve similar killing of *L. major*; this was confirmed by quantitative PCR. The combination of rapid accumulation and potent activity in the *Leishmania*-infected dermis indicates the potential of DNDI-0690 as a novel oral treatment for CL.

**Keywords:** cutaneous leishmaniasis, drug development, microdialysis, skin pharmacokinetics, rate of kill
Leishmaniasis is a poverty-associated infectious disease that has two main forms: visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL). While VL is almost invariably fatal if left untreated, CL is not life-threatening but causes disfiguring skin lesions associated with severe social stigma and psychological morbidity (1). The different types of CL have a wide geographic distribution and vary in causative Leishmania parasite species, which are transmitted to humans by infected female sand-flies. In the Middle-East, “Old World” L. major and L. tropica CL commonly present as local papules, nodules or ulcers that are mostly self-limiting, but often leave lifelong scars on the exposed skin areas. After healing, L. tropica CL can relapse into a persisting, chronic form called leishmaniasis recidivans. In Central and South America, “New World” parasite species of the Leishmania subgenus, such as L. mexicana, generally cause mild forms of CL, while more complicated forms involving the mucous membranes of nose, throat and mouth are observed in patients infected with the Viannia subgenus, for example L. braziliensis (2, 3). An additional cutaneous manifestation is post-kala azar dermal leishmaniasis (PKDL), a cutaneous sequela that can occur following the resolution of VL caused by L. donovani, characterised by widespread macular or papular lesions (3, 4). Worldwide, around 0.7 to 1.2 million new cases of CL occur every year and around 1 billion people are at risk, mostly those living in resource-poor environments (5). At present, treatment of CL is based on four drugs: pentavalent antimonials, miltefosine, amphotericin B and paromomycin. All of these have well-documented limitations of effectiveness, toxicity, cost or route of administration (6, 7). The Drugs for Neglected Diseases initiative (DNDi), a non-profit drug development partnership, has a strategy to deliver much-needed new drugs for CL. DNDi defines the optimal
Target Product Profile (TPP) of a new chemical entity for CL as follows: (i) activity against all species of *Leishmania* causing CL (> 15), (ii) minimum 95% clinical efficacy and minimal scarring after accelerated healing of the skin lesions, (iii) oral or topical formulation for a maximum of 7 or 14 days, respectively (iv) well-tolerated and safe in pregnancy and (v) cost under $5 per course (8). Whilst topical formulations hold potential for the treatment of simple, self-healing lesions, oral drugs could be used for cases with a higher risk of parasite dissemination; both routes of administration avoid the need for painful drug injections that are currently common (9).

Nitroimidazoles are a medically important class of antimicrobial agents with a broad spectrum of activity, including against protozoan parasites such as *Trichomonas vaginalis*, *Trypanosoma cruzi* and *Giardia* (10). The prototype molecule for this class, metronidazole, was discovered in the 1950s, and in recent years there has been a renewed interest in the therapeutic potential of nitroimidazoles, especially as novel antitubercular agents (11). Indeed, successful drug development efforts have resulted in the regulatory approval of delamanid (OPC-67683) for the treatment of multi-drug resistant tuberculosis (TB) by the European Medicines Agency (EMA) (12), while another bicyclic nitroimidazole compound, pretomanid (PA-824), is currently under investigation in Phase III clinical trials (13). In 2010, DNDi was granted access to a selected library of nitroimidazoles owned by the TB Alliance to speed up the development of novel therapies for neglected tropical diseases, including leishmaniasis. The antileishmanial activity of the nitroimidazooxazine DNDI-0690 (Figure 1) was first discovered in 2015; it is a structural analogue of DNDI-VL-2098 (14), a promising oral drug candidate for VL (15, 16) that was discontinued from further development due to toxicity issues identified during nonclinical CTA-enabling studies (6). Given its superior safety profile, potent *in vitro* activity (EC$_{50}$ = 0.17 μM)
and excellent in vivo efficacy (> 99 % at 12.5 mg/kg p.o. twice a day in hamster models of VL) (17, 18), the decision was made in 2018 to progress DNDI-0690 into in Phase 1 clinical trials for VL. Furthermore, DNDI-0690 demonstrated excellent in vitro activity against three Old and three New World cutaneous Leishmania strains (EC50 < 5 μM). In a mouse model of L. major CL, oral DNDI-0690 exerted a linear dose-response effect (ED50 = 5 mg/kg, ED90 = 21 mg/kg, maximal efficacy > 95 % for 50 mg/kg), while topical solutions applied directly to the skin lesion were < 50 % active (19).

With the clinical evaluation of DNDI-0690 for VL underway, important questions about the suitability, including appropriate PK and PD, of this nitroimidazole compound in the treatment of CL remain. The PK and PD properties required of drug to cure the two forms of leishmaniasis are not the same, due to (i) the different sites of infection that is the target for drug delivery (liver, spleen and bone-marrow versus dermal skin layers), (ii) possible differences in drug susceptibility between the causative parasites (L. donovani and L. infantum versus L. major, L. mexicana and other dermatropic Leishmania species) (20) and (iii) the potential impact of pathology on drug distribution. The aim of this study was to evaluate the PK and PD parameters of DNDI-0690 as part of efforts to develop much-needed new oral or topical drugs to treat CL. We therefore, determined the following properties of DNDI-0690: (i) in vitro drug disposition in skin upon topical dosing (Franz diffusion cells), (ii) in vitro protein-binding (BCA protein assay) and protein-binding corrected 50 % active drug concentrations against different CL-causing Leishmania species (fEC50), (iii) in vivo protein-free (e.g. pharmacologically active) drug exposure at the dermal site of infection (microdialysis) and (iv) in vivo time-kill kinetics of L. major and L. mexicana (bioluminescent parasite imaging).
Results

In vitro topical drug penetration

First, we evaluated topical drug penetration of DNDI-0690 into mouse skin in vitro using Franz diffusion cell permeation assays to investigate why the topical application of DNDI-0690 led to limited anti-leishmanial activity in murine models of CL (19). Table 1 shows skin distribution of topical DNDI-0690 into healthy and diseased but visibly intact skin (average nodule diameter 4.10 ± 0.72 mm) harvested from L. major–infected BALB/c mice. Six hours after application of a solution of DNDI-0690 saturated in ethanol:propylene glycol (EtOH:PG) (0.063 % W/V), around 99.5 % of the drug remained on the skin surface. Only a limited amount of drug (0.07-0.34 %) penetrated into the deeper layers of the (epi)dermis and 0.15-0.03 % passed through the skin, indicating poor dermal retention. There was no significant difference in drug quantity found in the different layers of the skin between L. major-infected and uninfected skin (p > 0.05).

In vitro anti-leishmanial drug activity corrected for protein binding

Second, in vitro 50 % effective concentrations (EC\textsubscript{50}) against Leishmania corrected for protein-binding (\textit{fEC\textsubscript{50}}) were calculated. This was done to enable comparison between in vitro PD measures (EC\textsubscript{50} value based on total drug concentrations in the drug assay medium) and in vivo PK parameters obtained by microdialysis (non-protein bound drug concentrations). Therefore, protein-binding in the in vitro assay medium (RPMI containing 10 % heat-inactivated foetal calf serum, HiFCS) was estimated using a rapid equilibrium method. Drug-protein binding in the medium was moderate: 45.8 % at 0.2 µM and 53.1 % at 1 µM DNDI-0690. The mean % binding
for DNDI-0690 (49.6 %) was used to determine \( fEC_{50} \), based on previously obtained \( EC_{50} \) values 132 (19) and described in Table 2.

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135 \textit{In vivo} skin pharmacokinetics

136 Third, we studied the \textit{in vivo} skin PK of DNDI-0690 in the \textit{L. major}-BALB/c mouse model using microdialysis. After administration of either a single oral (50 mg/kg) or topical (30 \( \mu \)l 0.064 % w/v) dose of DNDI-0690 to the infected mice, free drug concentrations in the infected dermis (target site), the uninfected dermis (off-target site) and plasma (systemic circulation) were determined (Figure 2). After oral dosing at 50 mg/kg, DNDI-0690 showed a gastrointestinal absorption delay of 2.5 hours before reaching an \( fC_{\text{max}} \) of 275.4 \( \pm \) 37.9 nM in the blood.

141 Systemic drug concentrations remained stable in the following 3.5 hours, indicating a plasma half-life (\( T_{1/2} \)) > 4 hours. Distribution volume (Vd) and elimination rate constant (Ke) values could not be estimated because no significant clearance of DNDI-0690 from plasma occurred within 6 hours of oral drug administration (\( t_{\text{last}} \)). The concentration of unbound DNDI-0690 in plasma was similar to unbound drug concentrations in infected and uninfected skin tissue and followed a comparable trend. Drug penetration from blood into skin tissue was high (\( fAUC_{0-6h, \text{tissue}} / fAUC_{0-6h, \text{blood}} > 80 \% \)) and maximal after 6 hours of oral dosing. However, DNDI-0690 concentrations and overall drug distribution to cutaneous tissues were increased in uninfected in comparison to infected skin (\( C_{\text{max}} = 365.3 \pm 47.1 \) nM \textit{versus} 263.7 \pm 28.0 nM; \( AUC_{0-6h, \text{tissue}} / AUC_{0-6h, \text{blood}} = 136.7 \% \textit{versus} 82.1 \%, \textit{respectively} \)). In contrast, after topical application of 50 \( \mu \)l DNDI-0690 saturated solution to the lesion, no drug was detected in the infected dermis within the following 6 hours. All results shown are corrected for \textit{in vitro} relative recovery (RR) of DNDI-
0690 from the microdialysis probe. The RR was 18.6 ± 2.3 % and independent of concentration under in vitro experimental conditions mimicking those in vivo.

In vivo antileishmanial pharmacodynamics

Fourth, the time-kill kinetics of DNDI-0690 were characterised in two BALB/c mouse models of CL using bioluminescent *L. major* and *L. mexicana* parasites. Topical activity of DNDI-0690 was not evaluated due to poor skin permeation and low efficacy when administered via this route. After oral dosing of DNDI-0690 (50 mg/kg once daily for ten days), rapid and complete clearance of *L. major* and *L. mexicana* from the infected mice was observed (Figures 3 and 4, respectively). A 10-, 100- and close to 1000-fold reduction in *L. major* parasite load (relative to organism burden in untreated mice at the same time-point) was observed by days 2, 6 and 10, respectively. The maximal killing of *L. major* (99.5 %) was achieved 24 hours after the 10th and final dose of DNDI-0690 (day 10). At this point, the efficacy of DNDI-0690 was comparable to that of the positive control drug intravenous liposomal amphotericin B (LAmB, 99.7%) in this model. An identical regimen of once daily 50 mg/kg DNDI-0690 resulted in a 100-fold reduction in *L. mexicana* parasite burden by day 2. After two oral doses, the bioluminescent signal in the DNDI-0690 treated group was indistinguishable from that of the mice infected with wild-type, not-bioluminescent parasites. The activity of DNDI-0690 against *L. mexicana* was maximal (99.4 %) and higher than that of LAmB (89.0%) at the end of treatment (day 10). Quantitative PCR was used to confirm the > 99 % reductions in parasite load for *L. major-* and *L. mexicana*-infected mice treated with oral DNDI-0690 compared to untreated controls (Figures 3 (C) and 4 (C), respectively).
Discussion

We have demonstrated the potential of DNDI-0690 as a novel treatment for CL by the oral route and the limited potential for its topical application. After oral administration at 50 mg/kg, DNDI-0690 is rapidly absorbed into the bloodstream and completely distributed to the skin, reaching near-maximal drug exposure at the site of action within 3 hours. At the dermal infection site, $f_{C_{\text{max}}}$ was lower (0.26 ± 0.03 µM) than $f_{EC_{50}}$ values for all tested Leishmania species (0.4 - 12 µM), indicating multiple doses could be needed to allow drug distribution to infected tissues and achieve cure. In vivo time-kill studies confirmed this was the case; in order to obtain a 100-fold reduction in lesion parasite load, 2 doses of 50 mg/kg were needed to clear L. mexicana ($f_{EC_{50}} = 0.96$ µM) versus 6 doses for L. major ($f_{EC_{50}} = 3.15$ µM). In both these bioluminescent Leishmania parasite CL mouse models, oral DNDI-0690 was as efficacious as the intravenous anti-leishmanial drug LAmB at the end of the 10-day treatment (> 99%). In contrast, topical administration of DNDI-0690 as a single application to the skin lesion did not result in measurable drug levels in the infected dermis. This may explain the low efficacy (< 50% reduction of lesion size and parasite burden determined by qPCR) of treatment via this route seen in earlier studies (19). These poor in vivo drug penetration kinetics, determined by skin microdialysis, were successfully predicted by in vitro Franz diffusion cell assays, which revealed the inability of DNDI-0690 to permeate the epidermis (> 99% drug was recovered from the skin surface). Such assays therefore save time and resources for the design and development of new topical formulations to treat simple CL (21).

To the best of our knowledge, this is the first time that skin microdialysis has been used to evaluate PK in Leishmania-infected mouse skin. The main technical advantage of this method for in vivo CL drug research is that it continuously measures protein-free (and, thus,
pharmacologically active) drug concentrations directly in the dermal interstitial fluid surrounding the parasitized macrophages (22). Voelkner and colleagues employed a similar approach to evaluating the proposed anti-leishmanial drug pyrazinamide, although this experiment was performed in healthy rats (23).

Interestingly, we observed differences in the PK of oral DNDI-0690 in *Leishmania*-infected and uninfected skin. Inflammation at the infection site in CL affects local drug distribution after intravenous administration of different formulations of amphotericin B (24, 25), as well as of topical drugs (26). Unbound DNDI-0690 concentrations in the dermal interstitial fluid could be lower in diseased than healthy skin, because while higher absolute amounts of drug may reach the skin tissue from the bloodstream (increased vascular permeability, vasodilation) (25), more drug could be bound to inflammatory proteins or engulfed by macrophages in the dermis. As neither protein-bound nor intracellular drug fractions are measured by microdialysis (27), this could explain the ultimately lower extracellular exposure of DNDI-0690 at the site of infection compared to uninfected counterparts. This finding illustrates the impact of the CL pathology on local drug distribution in the skin. Differences between amphotericin B and DNDI-0690 PK results could be related to the different sampling methodologies (skin necropsies and microdialysis, respectively).

A limitation of this work is the single, high dose of oral DNDI-0690 (50 mg/kg) that was used during the PK and PD experiments. Further dose fractionation studies are required to identify the PK/PD driver of efficacy in CL (28). Combined with extended PK studies in mice and man (different dose levels and, time points > 6 hours), available data on the susceptibility of six parasite species and strains to DNDI-0690 can be used to set a robust PK/PD target estimate to inform the design of optimal clinical dosing regimens.
In conclusion, the rapid oral absorption and potent activity of DNDI-0690 in skin lesions caused by *L. major* and *L. mexicana* support further development of this preclinical drug candidate as a new oral treatment for CL.
Materials and methods

Drugs and reagents. Oral DNDI-0690 was formulated in polyethylene glycol 400 (PEG400). The suspension was prepared in glass vials containing glass beads and sonicated (CamLab, Cambridge, UK) for 15 minutes before use. The dose levels and dosing frequency chosen were based on efficacy observed against VL (18) and CL (20). Topical DNDI-0690 was formulated as a saturated solution in propylene glycol-ethanol (PG-EOH, 1:1) to maximize permeation through the skin. The preparation was as follows. Excess of drug compound was added to a glass vial together with 1mL of PG-EtOH (1:1) and a magnetic stirrer. The vial was covered with aluminium foil and left at 34°C for 24 hours. An aliquot of this suspension was transferred to a vial and centrifuged for 15 min at 18,407 x g and 34°C after which the supernatant was transferred to a clean vial and stored at 4°C until drug administration. LC-MS/MS analysis confirmed the concentration of DNDI-0690 in this topical vehicle to be 0.063 % (W/V). Ringers solution was prepared at full strength (Sigma Aldrich) dissolved in 500 ml purified water) and autoclaved before use.

Parasite maintenance, animals and ethical statement. The bioluminescent strains *Ppy RE9H+L. major* Friedlin (MHOM/IL/81/Friedlin) and *Ppy RE9H+L. mexicana* M379 (MNYC/BZ/62/M379) were kindly provided by Elmarie Myburgh and Jeremy Mottram (University of York, UK). *L. major* JISH WT (MHOM/SA/85/JISH118), *Ppy RE9H+L. major* Friedlin and, *Ppy RE9H+L. mexicana* M379 were maintained in Schneider’s medium supplemented with 10% heat-inactivated foetal calf serum (HiFCS) and passaged weekly (1:10). Female BALB/c mice (age 6-8 weeks) were purchased from Charles River (Margate, UK) and left to acclimatise for 5 days upon arrival. One day prior to infection, the rump above the tail was shaven using electric clippers. Twenty-four hours later, low-passage late-stationary phase promastigote cultures were centrifuged at 900 x
g for 10 min at 4°C, counted using an improved Neubauer haemocytometer and re-suspended to 2 x 10^8 promastigotes per ml. Mice were subcutaneously injected in the rump with 200 µl of the suspension and randomly grouped (n=3-5). The mice were housed in a controlled environment of 55% relative humidity and 26°C and provided with tap water and a standard laboratory diet. All in vivo experiments were carried out under license (X20014A54) at the London School of Hygiene and Tropical Medicine (LSHTM) after discussion with the Named Veterinarian Surgeon and according to UK Home Office regulations.

**Bioanalysis of DNDI-0690 (LC-MS/MS).** All samples were analyzed using a Shimadzu Nexera X2 UHPLC/Shimadzu LCMS 860 at Pharmidex Pharmaceutical Services Ltd (Hatfield, UK). A mobile phase (0.4 ml/min) of water-0.1%formic (channel A) and acetonitrile-0.1%formic acid (channel B) was used to elute the sample compound from a Kinetex 5-µm XB-C18 column (2.1 mm by 50 mm at 50 °C; Phenomenex, UK). The mobile phase composition was initially 2% B, programmed to increase linearly to 95% B at 1.1 minutes after injection. After 0.7 minutes at 95 % B, the composition was returned to its initial 2% B at 1.8 minutes post-injection. DNDI-0690 was detected by monitoring the transition of the parent molecule (mass-to-charge ratio (m/z) 370) to the fragment resulting from electrospray ionization (m/z 198.2). Analyte concentrations were quantified against calibration standards prepared in matched control matrices, with aliquots of samples, blanks and standards being injected at 5 µl. Lower limit of quantifications ranged between 0.5ng/ml and 50ng/ml for the microdialysis and skin extraction samples, respectively (Supplemental material 1).

**In vitro drug binding.** The in vitro binding of the drug compounds to skin components was measured using the Rapid Equilibrium Dialysis single-use Device (Pierce Red Device, ThermoScientific). A 20 mM solution of DNDI-0690 in DMSO was used to spike RPMI-1640
medium supplemented with 10% HiFCS to a final concentration of 0.2 and 1 µM of DNDI-0690. Three hundred µL of the DNDI-0690 containing medium was transferred to the sample chamber and 550 µL of Ringers solution was added to the buffer chamber. This was done in triplicate for each DNDI-0690 concentration. The RED device was left to incubate in an orbital shaking incubator (200 rpm) at 34 °C for 4 hours. From each chamber, 50 µl aliquots were collected and matrix matched after which 2 volumes of ice cold ACN were added. After another 20 minutes, 100 µL of each mixture was centrifuged for 15 minutes at 21,130 x g at 4°C. The supernatants were assayed for the parent drug by LC-MS/MS.

**Franz diffusion cell permeation and drug disposition.** Female BALB/c mice (n=5) were injected subcutaneously with $4 \times 10^7$ *L. major* promastigotes above the tail. In time, a nodule developed at the injection site and when this reached 4 to 5mm the mice were sacrificed using CO₂. Two circular skin discs (approximately 15 mm diameter) were obtained per donor mouse; one containing the leishmaniasis nodule (average ± sd) that was collected from the lower dorsal area above the tail and another disc of unaffected skin that was collected from the area higher up the back of the mouse. Fat and muscle tissue were carefully removed using forceps and the skin was gently stretched on Whatman filter paper. The skin was placed between the greased donor and receptor compartment of the Franz cell with a narrow diameter (5 mm). PBS was sonicated for 30 minutes and added to the receptor compartment together with a small magnetic stirrer. The Franz cells were placed on the magnetic stirrer plate (800 rpm) in a warm water bath until the skin temperature reached a steady 34°C. Next, the DNDI-0690 saturated solution (30µL of 0.064% (W/V) DNDI-0690 in PG: EtOH (1:1)) was applied to the skin and 100 µl of receptor solution was replaced with fresh PBS at regular time intervals over a period of 6 hours and analysed by LC-MS/MS. At the end of the experiment, the cells were dismantled and...
the donor chambers of the Franz cells were washed with 1 ml of acetonitrile: water solution (ACN: H₂O (1:1)). Any drug remaining on the skin surface was removed using a clean dry cotton swab. The amount of drug in the washing liquid and the cotton swab was quantified using LC-MS/MS. To extract DNDI-0690 from the skin, the skin disc was homogenised in 1 ml of PBS as described above. 100 µL of this homogenate was protein precipitated using 300 µL of ice-cold ACN (100 %) and, centrifuged at 13,000 rpm for 30 mins at 4°C. An aliquot of the supernatant was diluted with an equal volume of water and stored for analysis by LC-MS/MS at -80°C. Together the amount of drug recovered from the skin surface, extracted from the skin and permeated through the skin was satisfactory when ranging from 70-110%.

Microdialysis system. MAB 1.2.4. Cu probes (Microbiotech, Sweden) with a 6kDa cut-off cuprophane membrane were used in vitro for recovery determination and in vivo for microdialysis. The cuprophane membrane of this concentric probe is 2 mm long and has an outer membrane diameter of 0.2 mm; inlet and outlet tubing consisted of fluorinated ethylene propylene (FEP) with lengths of 25 and 50 cm, respectively. A syringe pump (11 plus model 70-2208, Harvard Apparatus, USA) was used to circulate the perfusate (Ringers solution) at a flow rate of 2 µl/minute. Dialysates were automatically collected in glass vials (Thermo Fisher, UK) using a refrigerated fraction collector (MAB 85, Microbiotech, Sweden) at 30 minutes set intervals. For accurate measurement of in vivo free drug concentrations at the dermal site of action, raw microdialysis values were corrected for the loss of compound due to incomplete equilibration between the sampling medium and the perfusate and/or sticking of the drug to the outlet tubing of the microdialysis probe, expressed as the relative recovery value (22). Recovery rates for the microdialysis equipment were determined in vitro as follows: three probes were placed in a reservoir containing DNDI-0690 at concentrations of 30 or 120 ng/ml in
Ringers solution at 34 °C (mimicking in vivo skin temperature). The probes were perfused with Ringers solution at a flow rate of 2 µl/minute and microdialysates were collected every 15 minutes. All samples were analysed using LC-MS/MS after the addition of 10 µl acetonitrile (ACN) (1:3 ratio for 30 µl sample volume). Relative recovery (RR) was calculated as the ratio of the analyte concentrations in the microdialysate over the analyte concentration in the reservoir medium.

**In vivo** microdialysis. *L. major* JISH-infected BALB/c mice (n=6) with shaven rump and back were anaesthetised with 1.6 g/kg urethane (IP). Two hundred µl Ringers physiological solution was immediately administered via the neck scruff (SC) to prevent dehydration during long-term (6-8 hour) anaesthesia. Mice were placed on a temperature-controlled heating pad (Peco Services Ltd, Cumbria, UK) to maintain body temperature at 32 ± 2 °C. MAB 1.2.4. probes were inserted in the following positions using a 22 G needle: the dermal skin layer of the CL lesion on the rump, the dermal skin layer of the healthy control skin higher up on the back and the tail vein (Figure 5). To equilibrate the system and allow the skin and tail vein to recover from the probe insertion trauma, a stabilisation period of 30 minutes (23) of perfusion with Ringers solution at a flow rate of 2 µl/min was included before collecting samples. At the start of the pharmacokinetic experiment, half of the mice (n=3) received 50 mg/kg DNDI-0690 via oral gavage. This dosage has been shown to significantly reduce the lesion size (20). The other three mice received 30 µl of a 0.064 % (W/V) saturated solution (maximal driving force of 1) applied topically to the skin lesion on the rump of the mice. Samples were collected every 30 minutes at a flow rate of 2 µl/min. After the addition of 20 µl acetonitrile (1:3 ratio for 60 µl sample volume) samples were stored at – 80 °C before analysis by LC-MS/MS. Temperature, breathing...
pattern and behaviour of the anaesthetized mice were monitored constantly. At the end of the experiment, mice were culled by pentobarbital overdose.

Single-dose PK parameters were calculated by plotting the DNDI-0690 concentrations measured in the dialysate of the probe placed in the blood vene, the infected and uninfected skin over time. The fCmax for each matrix (blood, infected and uninfected skin) is the highest drug concentration reached in each respective matrix throughout the experiment. fAUC0-6h values for the blood and infected and uninfected skin were calculated using GraphPad Prism, version 7.02. Data are presented as mean and standard error of the mean (SEM).

Rate-of-kill by in vivo bioluminescence imaging. Thirty-six female BALB/c mice were purchased and prepared for infection as described above. Fifteen mice were injected with $4 \times 10^7$ stationary phase luciferase-expressing L. major Friedlin (Ppy RE9H+L. major Friedlin) promastigotes, fifteen were injected with luciferase-expressing L. mexicana M379 (Ppy RE9H+L. mexicana M379) (23) and six were infected with L. major JISH WT parasites that do not express luciferase. Upon appearance, nodule diameters were measured in two directions daily. When the size progressed to $6.73 \pm 1$ mm for the L. major Friedlin-infected mice, they were allocated into groups of five with similar average nodule diameters ($p>0.5$, One-Way ANOVA) and treatment was initiated. For the Ppy RE9H+L. mexicana M379, no lesions developed and treatment was started when the bioluminescence signal reached $5.02 (\pm 3.27) \times 10^6$ radiance/second. Each experiment included an untreated control (n=5), baseline control (L. major JISH WT, n=3), positive control (AmBisome®, iv, 25mg/kg, QAD, n=5) and a DNDI-0690 group (50 mg/kg, po, QD, n=5). A topical administration group was not included due to previously observed inactivity. The bioluminescent signal was measured prior to administration of the first drug dose and every other day thereafter until the baseline signal was reached. Ten
minutes before acquiring the bioluminescent signal, mice were injected with 150 mg/kg luciferin (d-luciferin potassium salt, Bertin Bioreagent), then anesthetized using 3% (vol/vol) gaseous isoflurane and placed in an IVIS Lumina II system (Perkin Elmer). Images were acquired 10 minutes after luciferin injection using LivingImage v4.3. A circular region of interest (ROI) encompassing the nodular area on the rump was drawn to quantify bioluminescence expressed as radiance (photons/second/cm²/sr). Background radiance was measured from mice infected with *L. major* JISH WT. Parasite burden in the skin was confirmed by DNA-based qPCR, as described earlier (24).

**Statistical analysis.** For the *in vitro* topical drug penetration experiment, differences between DNDI-0690 concentrations in healthy and infected skin were compared using student t-test (Prism v 7.02, Graphpad). To compare differences in qPCR parasite load in skin lesions, 1-way analysis of variance (ANOVA) followed by Dunnett’s multiple-comparison test was performed. A *P* value of <0.05 was considered statistically significant.

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References


Tables

Table 1. Disposition of topically applied DNDI-0690 in the skin of *L. major*-infected BALB/c mice using Franz diffusion cells. The total amount of DNDI-0690 per FDC recovered at the end of the experiment was considered 100%. The amounts of DNDI-0690 recovered from the different sites were expressed as a fraction of this amount. The table shows the average % (± SD), (infected n=5, uninfected n=3).

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<thead>
<tr>
<th>DNDI-0690 localisation</th>
<th>Uninfected skin</th>
<th>Infected skin</th>
<th>P-value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>On skin (DNDI-0690 in wash and cotton swab)</td>
<td>99.63 (± 0.39)</td>
<td>99.77 (± 0.19)</td>
<td>0.49</td>
</tr>
<tr>
<td>In skin (DNDI-0690 extracted from skin homogenate)</td>
<td>0.34 (± 0.39)</td>
<td>0.07 (± 0.07)</td>
<td>0.16</td>
</tr>
<tr>
<td>Through skin (DNDI-0690 in receptor fluid)</td>
<td>0.03 (± 0.05)</td>
<td>0.15 (± 0.15)</td>
<td>0.227</td>
</tr>
</tbody>
</table>

Table 2. Protein binding-corrected 50 % effective concentrations (*fEC50*) of DNDI-0690 against several cutaneous *Leishmania* species (n is the number of experiment repeats).

<table>
<thead>
<tr>
<th>CL- causing <em>Leishmania</em> species</th>
<th>n</th>
<th><em>EC50</em> (µM)</th>
<th><em>fEC50</em> (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. major</em> (MHOM/SA/85/JISH118)</td>
<td>1</td>
<td>4.56</td>
<td>2.28</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.94</td>
<td>3.97</td>
</tr>
<tr>
<td><em>L. tropica</em> (MHOM/AF/2015/HTD7)</td>
<td>1</td>
<td>1.41</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.38</td>
<td>1.19</td>
</tr>
<tr>
<td><em>L. aethiopica</em> (MHOM/ET/84/KH)</td>
<td>1</td>
<td>24.61</td>
<td>12.31</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&lt; 0.33</td>
<td>&lt; 0.165</td>
</tr>
<tr>
<td><em>L. mexicana</em> (MNYC/BZ/62/M379)</td>
<td>1</td>
<td>1.91</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&lt; 1.11</td>
<td>&lt; 0.56</td>
</tr>
<tr>
<td><em>L. panamensis</em> (MHOM/PA/67/BOYNTON)</td>
<td>1</td>
<td>0.77</td>
<td>0.39</td>
</tr>
<tr>
<td><em>L. amazonensis</em> DsRed2</td>
<td>1</td>
<td>&lt; 1.11</td>
<td>&lt; 0.56</td>
</tr>
</tbody>
</table>
**Figure legends**

**Figure 1.** The chemical structure of DNDI-0690.

**Figure 2.** Skin PK of DNDI-0690 in the *L. major*-BALB/c mouse model of CL after oral (left - each mouse had 3 probes inserted: tail vein, healthy and lesion skin) and topical (right - each mouse had 1 probe inserted: lesion skin) drug administration. Data represent protein-free drug concentrations (average concentration (nM) ± SD (n=3)), corrected for probe recovery.

**Figure 3.** Anti-leishmanial efficacy of oral DNDI-0690 (50 mg/kg, once daily for 10 days) in an ‘Old World’ CL model (*L. major* Friedlin REH infection of BALB/c mouse). (A) shows the parasite load, as indicated by *in vivo* imaging of bioluminescent parasites in the infected rump skin over time. (B) shows the bioluminescence signal on day 11 (24 hours after the last drug dose administration) and (C) confirms the parasite load on day 11 using qPCR. QD = once daily, QAD = once every 2 days, po=oral drug administration, iv= intravenous drug administration. * = p > 0.05.

**Figure 4.** Anti-leishmanial efficacy of oral DNDI-0690 (50 mg/kg, once daily for 10 days) in a ‘New World’ CL model (*L. mexicana* M379 REH infection of BALB/c mouse). (A) shows the parasite load, as indicated by *in vivo* imaging of bioluminescent parasites in the infected rump skin over time. (B) shows the bioluminescence signal on day 11 (24 hours after the last drug dose administration) and (C) confirms the parasite load on day 11 using qPCR. QD = once daily, QAD = once every 2 days, po=oral drug administration, iv= intravenous drug administration. * = p < 0.05. ** = p < 0.01.
Figure 5. Schematic of the experimental set-up of the \textit{in vivo} microdialysis in mice with CL.