Drug delivery; lessons to be learnt from *Leishmania* studies.

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Summary

Leishmaniasis is a disease caused by infection with the protozoan parasite *Leishmania* which is responsible for three main types of disease; cutaneous leishmaniasis, visceral leishmaniasis and mucocutaneous leishmaniasis which is related to the tissue tropism of the infecting species. This presents a major challenge to successful drug treatment, as a drug must not only reach antileishmanial concentrations in infected macrophages, the parasites' host cell, but also reach infected cells in locations specific to the type of disease. In this paper we discuss how studies using *Leishmania* have contributed to our knowledge on how drug delivery systems can be used to improve drug efficacy and delivery.

Keywords: Leishmaniasis, drug delivery systems, intravenous, non-invasive

Introduction

Leishmaniasis is a disease caused by infection with the protozoan parasite *Leishmania,* which is transmitted by female sandflies. The type of disease caused by the parasite depends on the infecting species and the host's immune response [*1*] but three main forms occur; cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL, Table 1). The World Health Organisation estimates that 350 million people, living in 98 countries, are at risk of contracting leishmaniasis, and each year approximately 1.5 million new cases of cutaneous and 500,000 of VL are reported. In terms of disease burden, leishmaniasis is responsible for 2,357,000 DALYs (Disability-Adjusted Life Years) lost due to ill effects caused by the disease.

Table 1 The main species responsible for leishmaniasis, their geographical distribution and site of the parasites within the body.

Type of leishmaniasis	**Main Species**	**Geographic Region**	**Tissue Tropism**
Visceral	*L. donovani, L. infantum, L. chagasi*	India, Nepal, Sudan, Brazil, Ethiopia	Disseminated in liver, spleen and bone marrow
Cutaneous	*L. major, L. tropica, L. mexicana*	Brazil, Colombia, Afghanistan, Iran, Saudi Arabia and Syria	Lesions form at the site of infected bite
Mucocutaneous	*L. braziliensis*	Brazil, Bolivia, Peru, Ethiopia	Mucosal tissue of mouth and nose

The *Leishmania* parasite has two distinct morphological forms in its life cycle, the intracellular amastigote in the mammalian host and the extracellular promastigote, which is transmitted by a sand fly vector. Infective promastigotes are deposited into the skin when an infected sand fly feeds. The promastigotes are taken up by phagocytes in the vicinity and transform into amastigotes within the parasitophorous vacuole. Over a period of 4-6 days, the amastigotes multiply inside the macrophage until its maximum capacity is reached and then the macrophage ruptures, releasing amastigotes which can infect new macrophages. The ability to control the infection depends on stimulating a protective immune response in the host. This ultimately results in activation of the cell's cytotoxic mechanisms, usually involving the production of reactive oxygen and nitrogen intermediates. Details on the specific responses involved, which vary between species, can be found in recent reviews [*2, 3, 4*]. The parasite's life cycle is completed when an uninfected sand fly takes a blood meal from the infected host.

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Currently there is no vaccine to prevent leishmaniasis in people therefore disease control depends on treating infected individuals or programmes which target the vector e.g. use of insecticide impregnated bed nets), vector control or the reservoir host e.g. the dog for VL. At present there are a limited number of drugs used in the treatment of leishmaniasis (Table 2) and many of the drugs are only suitable for use in certain geographical areas. For example, antimonials were the first line treatment for the majority of *Leishmania* infections for over 50 years but their use in treating VL is now limited due to the increasing incidence of drug resistance and relapse in endemic regions such as India and Nepal [*5,6*]. The introduction of miltefosine (MILT) for the treatment of VL was a major step forward as it was the first orally active drug. However there are already problems with a reduction in treatment efficacy, which could indicate that drug resistance is being introduced into the parasite population, possibly due to incorrect dosing by individuals [*7*]. Amphotericin B (AMB) is now the first line treatment for VL but it requires administration via the parenteral route and its use is limited by its inherent toxicity and high cost [*2*]. Although AMB resistance has been induced in laboratory strains [*8*], there is no evidence that it is present in field strains (*9*). Leishmaniasis is mainly a disease of the poor and so development of new drugs does not elicit the same interest for drug companies as other diseases. There is however a willingness to co-operate in providing drugs for leishmaniasis as the recent "London Declaration on Neglected Tropical Diseases" recently showed (http://www.who.int/neglected_diseases/London_Declaration_NTDs.pdf). One way to

improve drug treatment would be to use a drug delivery system to increase the efficacy of novel or existing drug. In this review we will discuss the variety of drug delivery systems that have been tested and demonstrate how studies on leishmaniasis have added to our knowledge on drug delivery.

Table 2 Drugs used in the treatment of leishmaniasis.

Drug	**Route**	**Dose**	**Adverse side effects reported**
Pentavalent antimonials	Intravenous/ Intramuscular	20 mgSb^V/kg for 28 days[a]	Vomiting and nausea. Widespread resistance
Pentamidine	Intravenous/ Intramuscular	4 mg/kg for 15 days alternative days[b]	Diabetes side effects
Paromomycin	Intramuscular	15 or 20 mg/kg for 21 days[c]	Renal Toxicity, Ototoxicity
Miltefosine	Oral	2.5 mg/kg for 28 days[d]	Poor compliance. Teratogenic
Amphotericin B	Intravenous	15 mg/kg for 30 days on alternative days, or 20 mg/kg/day for 20 days[e]	High costs. Nephrotoxicity
Sitamaquine	Oral	1.75, 2, 2.5 or 3 mg/kg/day for 28 days[f]	Abdominal pain, potential renal toxicity

The specific treatment regimen can vary for different *Leishmania spp* and their geographical location. Dosing regimens are shown from specific studies as an indication of treatment protocols used [[a]10; [b]11; [c]12; [d]13; [e]14, [f]15]

Drug delivery systems

The niche in which the *Leishmania* lives presents challenges to drug delivery, as the drug has to achieve antiparasitic levels in multiple sites and the specific area targeted depends on the species of *Leishmania*. For example in VL a drug must target parasites within macrophages in the spleen, liver and bone marrow, whereas in CL the drug must reach parasites in the cutaneous lesion(s). Thus a drug must cross multiple membranes to reach the intracellular

amastigote (Fig. 1) as the parasite is located within the parasitophorous vacuole within the macrophage. Imaging studies have shown the dynamic nature of these vacuoles and information on their biogenesis and during infection (*16*). These technical developments will aid in characterising delivery to the parasites within the parasitophorous vacuole but drug delivery to the *Leishmania* parasite still presents technical difficulties. Most analytical methods for drugs are based on high performance liquid chromatography and assessment of delivery to the parasite would require isolation of amastigotes, which may cause drug loss, and an assay method of a suitable sensitivity level to detect the drug present. Therefore most studies use reduction in parasite burdens as a measure of successful drug delivery. In most *in vivo* studies traditional pharmacokinetic parameters (e.g. distribution phase half-life; elimination phase half-life; area under the plasma concentration-time curve; volume of distribution, total body clearance) and drug levels at the targeted site are used to assess drug delivery. For example, antimonial drugs given by the intravenous route are only present in the blood for a short period of time as they have a short half life (absorption phase mean half-life of 0.85 h) and a rapid clearance (elimination mean half-life of 2.02 h, *17*), which would limit their uptake by the host cells and explain why multiple dosing is required for parasiticidal levels to reach the *Leishmania* parasites. Recent reviews discuss the problems associated with delivery of drugs using different routes of administration (18, 19, 20), the problems associated with drug delivery to specific sites or organelles within the body (20, 21) and drug deposition and uptake at these sites (21, 22). A drug delivery system can help achieve this aim as it directs more of the drug dose to tissues and away from the systemic circulation. Once the drug formulation has accessed cells at the site of uptake then the inherent pharmacokinetic properties of the drug within the formulation will influence its release into surrounding tissues (*23, 24*). Most drug delivery systems act as drug depots that decrease the release rate of the incorporated drug and therefore give more time for the drug to concentrate within the targeted cells. Macrophages, which are found in high concentrations in a number of locations in the body e.g. liver, lungs, spleen, play an important role in enhancing tissue uptake of particulate nanoformulations. Macrophages phagocytose particles from their immediate vicinity as part of their innate immune response and as a consequence act as a local drug depot. This means that the drug is directed directly to the *Leishmania* parasite in infected macrophages (*25, 26, 27*). Borborema *et al.,* 2011, demonstrated the advantages of this type of approach using a liposomal formulation of meglumine antimoniate They showed that using the carrier system reduced the 50% inhibitory concentration (IC_{50} value) value against the intracellular amastigote stage of *L. major* compared to the drug solution, from 93

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μM to 10.5 μM. Moreover, they also showed and that infected macrophages were more efficient than uninfected macrophages at taking up the liposomes (*28*). A drug delivery system can facilitate a reduction in the total drug dose and/or number of doses required, which is particularly important for a potentially toxic drug. For example, amphotericin B (AMB) that is associated with nephrotoxicity. This can be important for a drug that causes nephrotoxicity such as amphotericin B (AMB). This beneficial feature for drug delivery systems has been clearly demonstrated by the higher efficacy and lower toxicity of lipid formulations of AMB compared to AMB solution [*29]*. However these lipid formulations are prohibitively expensive for widespread use in endemic countries. This problem is being addressed by a World Health Organisation (WHO) initiative, which facilitated the donation of 445,000 vials of AMBisome for the treatment of VL.

Repurposing drugs originally designed for other clinical conditions gave new antileishmanial treatments. Thus AMB was originally developed for the treatment of fungal infections and MILT was originally in development for the treatment of cancer. Repurposing clinically approved drugs for treatment of leishmaniasis is an attractive approach as the majority of the required toxicity testing has already been completed, although additional testing would be required if a different mode of administration is used. Endemic countries often have traditional medicines that have been used for the treatment of leishmianiasis, and development of novel drugs from plant products has been investigated (30).

Intravenous delivery

The parenteral route is used for the majority of antileishmanial drugs as it ensures distribution of the drug to all sites of infection, however it also exposes non-target organs, which is particularly important for potentially toxic drugs. This route of administration has been used extensively in studies using a variety of drug delivery systems including liposomes, non-ionic surfactant vesicles (NIV), nanocapsule emulsions, nanodiscs and nanoparticles. Intravenous drug delivery has been very successful for treating visceral leishmaniasis as the liver and spleen, which are rich in macrophages, are the main organs for clearing particulates from the systemic circulation and the main sites of infection. Early studies by Abra and Hunt in a series of studies using radio-labelled liposomes showed that they delivered effectively increased delivery to the liver and spleen, and that dose, size and dosing regimen influenced delivery (*31, 32, 33*)

Liposomes are synthetic vesicles, prepared using phospholipids that form a natural bilayer. The exact composition of liposomes can vary and often includes phosphatidylcholine (PC) and cholesterol. By assembling the liposomes in conjunction with an aqueous solution, the compound is encapsulated in the inner core of the lipid bilayer. When the liposome comes in to contact with a cell membrane it may fuse with itor be taken up by phagocytosis, thus delivering the drug solution inside the cell. Vesicle characteristics such as composition, size, surface charge and drug loading all have important influences on drug delivery (*31, 32)* Roychoudhury *et al. (34*) prepared liposomes containing sodium stibogluconate (SSG) from PC and stearlyamine (PC-SA) or PC and cholesterol (PC-Chol) using sonication followed by centrifugation at 60,000 x g to remove unentrapped drug, a method that would not adapt well to large-scale manufacture and has been shown to damage the vesicle bilayer (*35*). Single-dose treatment of mice infected with *L. donovani* strains that had different inherent susceptibilities to SSG with PC-SA liposomes containing SSG (PC-SA-SSG, 12 mg Sb^{v}/kg) resulted in a significant reduction in liver, splenic and bone marrow parasite burdens (>84%) whereas similar treatment with PC-Chol liposomes containing SSG (PC-Chol-SSG) only caused a significant reduction in liver parasite burdens. The advantage of using a carrier system was clearly demonstrated in this study as similar treatment with SSG solution at a dose of 300 mg Sb^{v}/kg only significantly affected liver parasite burdens in mice infected with a SSG susceptible strain. Drug uptake studies in parasites showed that significantly higher levels of Sb were present in amastigotes recovered from infected macrophages treated with PC-SA-SSG compared with PC-Chol-SSG or SSG solution. The entrapment efficiencies quoted for the two formulations were similar, indicating that both type of carriers would deliver a similar amount of drug to cells, therefore different in drug delivery may reflect differences in the rate uptake for the two type of liposomes (*36*). PC can interact with phosphatidylserine (PS) residues present on the cell membranes and this could improve uptake of PC-SA liposomes [*37*].

We have shown that non-ionic surfactant vesicles (NIV), which are analogous to liposomes, but contain a non-ionic surfactant instead of a phospholipid, are very effective at delivering various antileishmanial drugs (SSG, amphotericin B and paromomycin) in rodent and canine models of visceral leishmaniasis [*38, 38, 40, 41*]. The enhanced activity compared to drug solution alone was associated with the ability to favourable alter the *in vivo* pharmacokinetics of the drug. For example, treatment of dogs with SSG-NIV prevented the rapid elimination phase associated with free SSG treatment, resulting in significantly higher antimony levels in

the liver and spleen (*39*). Similar treatment with SSG-NIV had no adverse effect on lesion development in *L. major* infected mice (unpublished data), probably because the formulation did not target the drug to the skin parasites. More recent studies clearly demonstrated this effect. Iintravenous treatment with a NIV formulation containing luciferin did not enhance delivery of luciferin to luciferase-expressing *L. major* parasites within the footpad of a mouse, but did enhance delivery of the substrate to luciferase-expressing *L. donovani* parasites located within the liver of a co-infected mouse (Fig. 2).

Kansal et al. [*42*] showed that a nanocapsule emulsion (NC) containing doxorubicin (NC-DOX) had a 1.75 fold higher uptake if PS was anchored on to its surface compared to non-PS containing NC-DOX. The high drug levels within amastigotes after PC-SA-SSG compared to PC-Chol-SSG treatment could be related to lower inhibition of macrophage function, a factor that could be explored by infecting macrophages using different parasite: macrophage ratios and determining the effect on SSG uptake.

Modifying the surface of the delivery vehicle to increase the time it remains in the systemic circulation or by incorporating molecules, which target surface receptors on the target cell, can increase the efficacy of a drug formulation. This has been exploited by incorporation of polyethylene glycol (PEG) into liposomes to produce 'long circulating' or 'stealth' liposomes that are more likely to be taken up by tissues [43]. Integrating dendrimers, which interact with MHC class II molecules, into amphotericin B liposomes increased their uptake by phagocytic cells and intravenous treatment of *L. major* infected mice with this formulation was more effective at treating skin lesions compared to liposomal AMB alone. Interaction with MHC class II molecules was confirmed to be important as incorporation of a dendrimer that targeted a random peptide did not increase the efficacy of liposomal AMB [44] An unexpected side effect of this formulation was its ability to boost host immune responses, leading to enhanced interferon gamma production by *L. major* specific splenocytes. Infection with *Leishmania* suppresses immune responses in susceptible individuals both at the local (i.e. infected macrophage) and whole body level, so production of an immunotherapeutic drug formulation would be ideal for leishmaniasis [*45*].

This combined immunotherapeutic approach was recently tested using a liposomal formulation of resiquimod [*46*], a derivative of imiquimod, which is an FDA-approved Toll-like receptor 7/8 agonist, and also has FDA approval for cutaneous use. Resiquimod liposomes were prepared by dissolving the formulation constituents in methanol/chloroform

mix, rotary evaporation was then used to remove the solvents and drug loaded liposomes were formed by hydration with water. Freeze-thaw cycles were used, presumably to improve drug entrapment as a reduction in size was achieved by passing the liposome suspension through an extruder fitted with an 80 nm polycarbonate membrane. Unentrapped drug was removed from the resulting suspension by passing down a PD-10 column and the liposomes were lyophilised in the presence of sucrose as the cryoprotector. On reconstitution, liposomes with a mean vesicle size of 75 ± 31 nm and an entrapment efficiency of 7% was obtained. Intravenous treatment with a single dose of resiquimod liposomes (0.38 mg/kg, assuming a 20 g mouse weight) caused a significant reduction in splenic, liver and bone marrow *L. donovani* parasites compared to treatment with the carrier alone but the drug formulation was not as effective as single dose treatment with SSG (500 Sb^v/kg). Determination of host immune responses showed that resiquimod liposome treatment was associated with enhanced interferon gamma (IFN-γ) and interleukin 10 (IL-10) production by splenocytes activated with specific antigen compared to control or carrier alone groups. These two cytokines act antagonistically as IFN-γ production stimulates macrophages to kill *Leishmania* and is associated with protection against *L. dovovani* infection whereas IL-10 suppresses macrophage activation and its production is associated with susceptibility [*47*]. However the relative local concentration of each cytokine at the site of infection will be important in determining whether the immunostimulatory effects aid parasite clearance or not.

Nanodiscs or nanodisks (ND) are structurally similar drug carriers to liposomes and have a phospholipid bilayer integrated with apolipoprotein [*48*]. They are typically less than 200 nm in size have a hydrophobic core instead of an aqueous core, which is surrounded by apolipoprotein. This is beneficial from a drug delivery point of view as the ND are soluble in the aqueous conditions of the bloodstream [*49*]. For this reason, ND are have been used to solubilise and improve the delivery of the relatively insoluble AMB. In a murine model of cutaneous leishmaniasis, intravenous treatment with four doses of AMB-ND (5mg/kg, days 4, 7, 14 and 21) completely cleared *L. major* parasites and lesions in BALB/c mice. Treatment with a comparable dose of liposomal AMB (AMBisome) reduced parasite burden and lesion size but did not achieve cure. Moreover, there was no appreciable change in cytokine levels in the AMB-ND treated mice which indicates that the clearance was not associated with a T helper cell response switch and suggests that a nanodisc based AMB treatment could be suitable for immunocompromised patients [50]. The superior efficacy of the ND could be

related to the way AMB is incorporated into the ND. AMB causes ND bilayer interdigitation and in treated *Leishmania* cells there would be a reduction in bilayer thickness of the host/parasite membrane bilayer, so that only 1 AMB molecule spans the membrane bilayer, and as AMB molecules self-associate, a pore formed from 8-12 AMB would form. This pore results in leakage in the target cell membrane resulting in death of the host cell/parasite cell [51].

Conjugating drugs to polymeric nanoparticles is another strategy used to deliver drugs to particular target sites. Polymeric nanoparticles have an advantage over lipid formulations as their production costs are usually lower and the shelf life at room temperature is extended [*52*]. Gaspar *et al.* [53] and Paul *et al.* [*54*] showed that polyalkylcyanoacrylate (PACA) primaquine or polymethacrylate (PMMA) pentamidine nanoparticles respectively could be used to enhance drug delivery. Poly(lactide-co-glycolide) (PLGA) nanoparticles have been used to improve the delivery of AMB in a number of studies. For example, Nahar and Jain [*55*] produced PLGA nanoparticles conjugated to AMB (AMB-NP) which had a size of less than 200 nm and a polydispersity index (PDI) below 0.16. Additionally, inclusion of PEG to couple PGLA to mannose improved their uptake by macrophages and efficacy against *L. donovani ex vivo* amastigotes. An *in vitro* study showed that saponin loaded PLGA-nanoparticles were active against axenic and *ex vivo* amastigotes of *L. infantum* and confocal microscopy allowed visualisation of saponin-loaded nanoparticles uptake by *L. donovani* infected macrophages [56]. The same group recently characterised the delivery of AMB-PLGA nanoparticles against *L. infantum* parasites and several fungal species a showed that the formulation was was either equivalent or more efficacious than AMBisome or Fungizone against promastigotes and amastigotes [*57*].

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Non-invasive drug delivery

Ideally, any new drug formulation developed for the treatment of leishmaniaisis should be administered by a non-invasive route as it removes the requirement for hospitalisation, improves patient compliance and removes other risks e.g. occupational risk of infection or environmental hazards associated with disposal of contaminated sharps. There are a number of routes that can be used [*58*] but the three that are probably most relevant for leishmaniasis are oral, pulmonary and topical.

Oral delivery is the preferred method for patients and clinicians, as it does not require hospitalization of the patient. Developing oral formulations of current chemotherapy options

is attractive as it can help reduce the effective dose required, helping to reduce both side effects and the cost of treatment. However this route offers its own challenges as the gastrointestinal tract provides harsh physicochemical conditions. In addition any drug taken up in the gut has to undergo first pass metabolism in the liver, where it is exposed to enzymes [*59*]. Although patient compliance is higher for oral formulations, a lack of treatment supervision can lead to patients not completing the full course of treatment. This is a major issue as it may facilitate the development of drug resistance in *Leishmania*. MILT is the only oral drug used in the clinical treatment of leishmaniasis and it was originally developed as an anticancer agent. It is highly effective against VL, giving cure rates of 94%. But recent evidence in India indicates that relapse rates are now higher, even though there was no apparent increase in MILT resistance in parasites isolated from VL patients before, and after, treatment. Parasites isolated from post dermal kala patients do exhibit increased resistance to MILT [*60*] and in Nepal the number of VL patients are not responding to MILT treatment is increasing [*61*].

Imipramine, clinically used to treat treat depression by the oral route, has recently been shown to be effective against *L. donovani*. Repurposing drugs for other clinical indications has been suggested for a some time [*62*] but it is likely to require public funding for leishmaniasis.

A number of different delivery systems have been used to formulate oral drug formulations [63] and in leishmaniasis the main drug used in studies is AMB as it can also be used to treat fungal infections. AmB is poorly soluble and susceptible to degradation in the gut. As such, parental administration is necessary. An oral formulation stabilising AmB and improving solubility would be beneficial. A 5-day oral treatment protocol using carbon nanotubes covalently linked to AMB (f-CNT-AMB) at a dose of 15 mg AMB/kg/day resulted in a 98% reduction in splenic parasite burdens in *L. donovani* infected hamsters [*64*]. Previous studies had shown that the f-CNT carrier alone had antileishmanial activity, although the intraperitoneal route was used in the original study. It was suggested that induction of host immunity rather than induced cytotoxicity was responsible for the antileishmanial activity as a slight inflammatory response was noted at the injection site in mice. *In vivo* studies showed that the carrier did not affect creatine or alkaline phosphatase, sodium glucose phosphatase levels, markers associated with toxicity [*65*]. The f-CNT-AMB formulation was stable six month after storage at room temperate but further studies are intended to improve the

formulation. Studies using Caco-2 cell monolayers have shown that f-CNT can cause reversible modulate tight junction formation, which would be required for transport across the gut epithelial barrier. In addition f-CNT had the ability to down regulate the activity of P glycoprotein efflux activity, which would help to increase the bioavailability of any drug incorporated into the carrier system [*66*]. CNT have also been shown to activate reactive oxygen species but this activity was associated with cytotoxicity in treated keratinocytes [*67*], so may not be important in the improved f-CNT formulations.

Nanoemulsions are mixtures of two normally immiscible liquids that are usually stabilised by using a surfactant, which can form a polymer shell around the outside of the emulsion to prevent coalescence. Formulating the emulsion as oil-in-water allows the solubilisation of poorly aqueous soluble drugs, such as AMB, and protects them from degradation. The first example of this formulation for treatment of VL was reported in 2004 [68]. IC0-010, a lipid emulsion formulation of AMB currently being developed by iCo-therapeutics, was granted orphan drug status by the US Food and Drug Administration [*69*]. The formulation contains a mono- and di-glycerides and d-α-tocopheryl polyethylene glycol succinate, which form a self-emulsifying structure that forms droplets in intestinal conditions at 37°C. The formulation has been shown to be highly effective in a murine model of VL and is stable at temperatures expected in tropical environments. Less developed formulations of AMB have been produced by other researchers. For example, a nanoparticle formulation of AMB was shown to be more effective that Fungizone in a murine model of VL but further work is required to improve its *in vivo* activity [*70*].

Although pulmonary delivery is used mainly as a non-invasive route to target drugs directly to the lungs it has also been used to deliver drug systemically as the lung epithelium is very close to the blood circulation. Drug formulations are aerosolised, normally using an inhaler/nebuliser. The aerosol droplet size has a major influence on deposition of the inhaled drug formulation within the lungs. Ideally droplets need to have a mass median aerodynamic diameter (MMAD) of 0.5-5 μm [*71, 72, 73*]. If the droplets are too large they will not reach the aveoli as they are trapped in the upper airway and then swallowed as part of the normal lung clearance mechanisms, and if they are too small then the drug formulation would be removed from the lungs when the patient breathes out. There are a number of excellent reviews on type of nebulisers and factors affecting lung deposition. The mucus lining the lungs can impede the uptake of drug formulations and the presence of enzymes in lung lumen can lead to drug metabolism, which could inactivate the drug before its up take up by cells

within the lung epithelium. The lungs have a larger macrophage population and these cells have a major role in taking up particulates. This means that nanocarriers are likely to be cleared by these cells, providing a deposition site for the drug, which would be released from the carrier at the site of uptake during phagocytosis. Imaging studies have shown that there is one resident macrophage for every three aveoli and that these cells remain sessile even when challenged with a bacterial pathogen, This could indicate that these cells are unlikely in movement of a 'intracellular drug depot' to a site of infection to favour local drug delivery [*74*]. However dendritic cells are mobile and they could be responsible for trafficking drug to site of infection/local lymph nodes as part of their normal immune responses [*75*]. Technical difficulties make it difficult to determine if intact drug carriers access the systemic circulation after uptake in the lungs, therefore delivery of inhaled drug formulations to sites other the lungs is mainly based on determination of drug/label incorporated into the carrier system. This approach has been used in the treatment of lung conditions such as Invasive pulmonary aspergillosis (IPA). For example prophylactic treatment of neutropenic patients with haemolytic disease with liposomal amphotericin B was tested. The study showed that treatment significantly reduced the incidence of IPA and prevented the renal toxicity associated with intravenous AMB treatment. Carter *et al.* [*76*] have shown that pulmonary route is not only suitable for lung conditions but it can also be used to target liver conditions. Thus treatment with a NIV formulation of AMB resulted in a significant reduction in liver parasite burdens in a murine model of VL and the same formulation was effective at reducing *Aspergillus* levels in the lungs. This route is unlikely to be suitable for deeper tissue sites as the formulation failed to significantly affect splenic and bone marrow parasite burdens in *L, donovani* infected mice and had no significant effect on lesion progression in mice infected with *L. major.* Imaging studies indicated that the lack of effect was due to an inability to enhance delivery to the footpad of mice infected with luciferase-expressing *L. major* (Fig. 3).

Topical treatment is particularly attractive for the treatment of CL as the drug is applied directly to the lesion, however, it may be less efficacious against VL. The skin presents a formidable challenge to drug delivery as it is adapted to prevent entry of potentially harmful agents [*77*]. One of the drugs commonly used in topical treatments for CL is the aminoglycoside PMM, which was first tested by El-On and colleagues in the 1980's [*78*]. Studies using liposomal formulations of PMM indicated that the vesicle improved drug delivery into the skin but the levels achieved (5-7% of the applied dose) were still very low [*79*]. Another study using liposomal paromomycin had higher skin penetration (15% of the

applied dose). Four weeks treatment using liposomal PMM at 50mg/dose twice a day cured mice [*80*]. However it is possible that mice ingested some of the drug formulation as we have found that mice clean away any formulation applied to the skin, even if it contains denatonium benzoate, a compound used to deter ingestion [*81*].

Future perspectives

The ability to successful treat *Leishmania* depends on not only identifying an effective drug, it is also important to deliver that drug at parasiticidal levels into infected macrophages, which can reside at multiple sites within the body. Advances in non-invasive imaging techniques [*82*] and the production of novel *Leishmania* strains expressing reporter genes e.g. green fluorescent protein (GFP), cherry red fluorescent protein or luciferase [83], have allowed detailed studies on disease burdens and pharmacokinetic studies at both the cellular level and in individual animals. Selection of the most appropriate label for experimental studies is essential. For example, GFP-labelled parasites allow direct visualisation of parasites without requiring a substrate (luciferin), which could be more beneficial for *in vitro* assays, however, for *in vivo* studies where parasites are located in deeper tissues rich in blood (e.g. the liver), then luciferase-expressing parasites may be more appropriate. Tissues can absorb the light emitted by fluorescent dyes and light scattering can mean that a lot of the light does not reach the detector or does not indicate the correct localization of parasites. Bioluminescence gives a stronger signal but it is not without its problems. Luminescence depends on delivery of luciferin to luciferase-expressing cells. Luciferin has a large volume of distribution after the recommended intraperitoneal injection but a short *in vivo* half-life, meaning that imaging is usually carried out within 30 min of dosing. Bioluminescence is also dependent on the presence of oxygen, therefore there will be a lo/no signal if if hypoxia is present at the site of the luciferase-expressing cells. The development of better gene reporter systems and continued development of imaging systems that allow integration of information from different types of imaging systems (e.g. fluorescence, bioluminescence, radioisotope imaging and X-ray-based computed tomography) means that a better understanding of the disease processes and treatment outcome is possible. It is now possible to have high throughput screening of compounds against the more clinically relevant intracellular amastigote stage of *Leishmania* rather than the promastigote stage [84] and it is possible to determine the effect of drug treatment on parasite burdens in multiple sites within the same animal at different times post-treatment, to get a better indication of how altering treatment regimens effects parasite numbers. It is also possible to determine how effective a drug

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delivery system is at delivering a drug to different sites in the body, allowing rational design a drug delivery system so its effect on its delivery capabilities can be assessed. We have shown that it is possible to monitor drug delivery to multiple sites within the same animal using this type of imaging technology (Fig. 2). Eventually these types of studies will provide the data to mathematically model drug delivery so that the knowledge gained from other specialties e.g. fluid mechanics can be integrated so that it may eventually be possible to study *in vivo* drug delivery using very few animals and systems specific to the species being treated. Researchers need to keep in mind scale up parameters of their formulations at the early stage of development and try and use constituents of pharmaceutical grade where possible. Quite often experimental methods contain steps that are not practical for large-scale manufacture and considerations like these early on can save time further down the line. All our formulation studies have suggested that drug loading is one of the most important parameters in formulation efficacy, a factor strongly influenced by drug solubility. Therefore, chemical modification of active compounds to increase their aqueous solubility is an important area of research. In addition any drug development programme must include screening against recently isolated field strains in their studies as it is possible that by the time the drug formulations have gone through clinical trials it may be inappropriate for use in endemic countries. We developed a NIV formulation of sodium stibogluconate, which was effective a single intravenous dose in rodent VL models, but funding for this project stopped once it became apparent that antimony resistance was widespread in India. There are number of studies determining the molecular basis for resistance to antileishmanial drugs as knowing how the mechanism may allow the development of novel therapies that can block the mechanism(s) and turn a clinically ineffective drug back into an effective treatment. For example, resistance to PMM in *L. donovani* was related to increased membrane fluidity accompanied with decreased intracellular drug accumulation and was associated with increased expression of ATP-binding cassette (ABC) transporters (MDR1 & MRPA) [*85*] and the ABC transporter gene *MRPA* was amplified in antimony resistant field isolates of *L. donovani* [86]. ABC transporters also mediate drug resistance in cancer cells [87], therefore an understanding of how they operate in *Leishmania* is relevant to other clinical conditions.

Leishmania may be a species that is a 'neglected tropical disease' but this pathogen can provide fundamental information on drug delivery that is relevant to diseases that reside in similar tissues/organs within the body. Therefore funding research into this pathogen is easy to justify, even if it is a disease associated with poverty.

Executive summary

- Leishmaniasis: current drugs used and their limitations
- Drug delivery systems: the role of drug delivery systems in modifying drug delivery to the *Leishmania* parasite are discussed: including vesicles, nanocapsule, nanodiscs, nanoparticles, nanotubes, nanoemulsions
- Intravenous delivery: novel drug formulations given by the intravenous route are discussed
- non-invasive drug delivery: novel drug formulations given by the oral, pulmonary and cutaneous routes are discussed
- Future perspectives: co-ordinating data obtained using new imaging technologies along with engineering disciplines such as fluid dynamics may aid in the design of better drug delivery system

References

1. Heyneman D. Immunology of leishmaniasis *Bull World Health Organ*. 44(4), 499-514 (1971).

2. Moore EM, Lockwood DN. Treatment of visceral leishmaniasis. *J Glob Infect Dis.* 2(2), 151-8 (2010).

3. Okwor I, Mou Z, Liu D, Uzonna J. Protective immunity and vaccination against cutaneous leishmaniasis. *Front Immunol.* 29(3), 128 (2012).

4. de Oliveira CI, Brodskyn CI. The immunobiology of *Leishmania braziliensis* infection. *Front Immunol*. 8(3), 145 (2012).

5 Rijal S, Chappuis F, Singh R *et al*. Treatment of visceral leishmaniasis in south-eastern Nepal: decreasing efficacy of sodium stibogluconate and need for a policy to limit further decline. *Trans R Soc Trop Med Hyg*. 97(3), 350-354 (2003).

6. Musa A, Khalil E, Hailu A *et al.* (SSG) & paromomycin combination compared to SSG for visceral leishmaniasis in East Africa: a randomised controlled trial. *PLoS Negl Trop Dis*. 6(6), (2012).

7. Sundar S, Singh A, Rai M *et al.*Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use.Clin Infect Dis. 55(4):543-50 (2012

8. García-Hernández R, Manzano JI, Castanys S, Gamarro F. Leishmania donovani develops resistance to drug combinations. *PLoS Negl Trop Dis*. 6(12), (2012).

9. Prajapati VK, Mehrotra S, Gautam S, Rai M, Sundar S. In vitro antileishmanial drug susceptibility of clinical isolates from patients with Indian visceral leishmaniasis--status of newly introduced drugs. *Am J Trop Med Hyg*. 87(4), 655-657 (2012).

10. Ritmeijer K, Dejenie A, Assefa Y *et al*. A comparison of miltefosine and sodium stibogluconate for treatment of visceral leishmaniasis in an Ethiopian population with high prevalence of HIV infection. *Clin Infect Dis* 43(3), 357–364 (2006).

11. Das VN, Siddiqui NA, Pandey K,*et al*. A controlled, randomized nonblinded clinical trial to assess the efficacy of amphotericin B deoxycholate as compared to pentamidine for the treatment of antimony unresponsive visceral leishmaniasis cases in Bihar, India. *Ther Clin Risk Manag*. 5(1), 117-124 (2009).

12. Musa AM, Younis B, Fadlalla A, *et al.* Paromomycin for the treatment of visceral leishmaniasis in Sudan: a randomized, open-label, dose-finding study. *PLoS Negl Trop Dis*. 4(10), e855 (2010).

13. Sundar S, Chakravarty J, Rai VK, Agrawal N, Singh SP, Chauhan V, Murray HW. Amphotericin B treatment for Indian visceral leishmaniasis: response to 15 daily versus alternate-day infusions. *Clin Infect Dis*. 45(5), 556-561 (2007).

14 Rahman M, Ahmed BN, Faiz MA *et al*. Phase IV trial of miltefosine in adults and children for treatment of visceral leishmaniasis (kala-azar) in Bangladesh. *Am J Trop Med Hyg*. 85(1), 66-69 (2011).

15 Wasunna MK, Rashid JR, Mbui J *et al*. A phase II dose-increasing study of sitamaquine for the treatment of visceral leishmaniasis in Kenya. *Am J Trop Med Hyg*. 73(5), 871-876 (2005).

16. Real F, Mortara RA. The diverse and dynamic nature of Leishmania parasitophorous vacuoles studied by multidimensional imaging. *PLoS Negl Trop Dis*. 6(2), e1518 (2012)

17**.** Chulay JD, Fleckenstein L, Smith DH. Pharmacokinetics of antimony during treatment of visceral leishmaniasis with sodium stibogluconate or meglumine antimoniate. *Trans. R. Soc Trop Med Hyg* 82(1), 69-72 (1988).

18. Nino M, Calabrò G, Santoianni P.Topical delivery of active principles: the field of dermatological research. *Dermatol Online J*. 16(1):4. (2010).

19. Zarogoulidis P, Chatzaki E, Porpodis K *et al*. Inhaled chemotherapy in lung cancer: future concept of nanomedicine. *Int J Nanomedicine*. 7:15, 51-72, (2012).

20. Diab R, Jaafar-Maalej C, Fessi H, Maincent P. Engineered nanoparticulate drug delivery systems: the next frontier for oral administration? *AAPS J*. 14(4), 688-702 (2012).

21. Mishra N, Yadav NP, Rai VK, Sinha P, Yadav KS, Jain S, Arora S. Efficient hepatic delivery of drugs: novel strategies and their significance. *Biomed Res Int.* 382184 (2013).

22. Sakhrani NM, Padh H.Organelle targeting: third level of drug targeting. *Drug Des Devel Ther*. 17;7, 585-99 (2013)

23. Benet LZ. Predicting Drug Disposition via Application of a Biopharmaceutics Drug Disposition Classification System. *Basic Clin Pharmacol Toxicol*. 106(3), 162–167 (2010).

24. Burton PS, Goodwin, JT, Vidmar TJ, Amore BM. Predicting Drug Absorption: How Nature Made It a Difficult Problem *J Pharmacol Exp* Ther. 303, 889-895 (2002).

25. Date AA, Joshi MD, Patravale VB. Parasitic diseases: Liposomes and polymeric nanoparticles versus lipid nanoparticles. *Advanced Drug Delivery Reviews* 59(6), 505–521 (2007).

26. Romero EL, Morilla MJ. Drug delivery systems against leishmaniasis? Still an open question. *Expert Opin. Drug Deliv.* 5(7), 805–823 (2008).

27. Espuelas S. Delivery systems for the treatment and prevention of Leishmaniasis. *Gaz.* Med. Bahia 79 (supl. 3), 134–146 (2009).

28 ????????? Borborema *et al.,* 2011????????????????????????

29. Balasegaram M, Ritmeijer K, Lima MA *et al*. Liposomal amphotericin B as a treatment for human leishmaniasis. *Expert Opin Emerg Drugs*. 17(4), 493-510 (2012).

30. Brito AM, Dos Santos D, Rodrigues SA, Brito RG, Xavier-Filho L. Plants with anti-Leishmania activity: Integrative review from 2000 to 2011. *Pharmacogn Rev*. 7(13), 34-41 (2013).

31. Abra RM, Hunt CA. Liposome disposition in vivo. III. Dose and vesicle-size effects. *Biochim Biophys Acta*. 23;666(3), 493-503 (1981).

32. Abra RM, Bosworth ME, Hunt CA. Liposome disposition in vivo: effects of pre-dosing with lipsomes. *Res Commun Chem Pathol Pharmacol*. 29(2), 349-60 (1980).

33. Abra RM, Hunt CA. Liposome disposition in vivo IV: the interaction of sequential doses of liposomes having different diameters. *Res Commun Chem Pathol Pharmacol*. 36(1), 17-31 (1982).

34. Roychoudhury J, Sinha R, Ali N. Therapy with sodium stibogluconate in stearylamine-bearing liposomes confers cure against SSG-resistant Leishmania donovani in BALB/c mice. *PLoS One*. 6(3), e17376. (2011).

35. Heeremans JL, Gerritsen HR, Meusen SP *et al.*. The preparation of tissue-type Plasminogen Activator (t-PA) containing liposomes: entrapment efficiency and ultracentrifugation damage. *J Drug Target*. 3(4), 301-310 (1995).

36. Dey T, Anam K, Afrin F, Ali N. Antileishmanial activities of stearylamine-bearing liposomes. *Antimicrob Agents Chemother*. 44(6), 1739-1742 (2000).

37. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol*. 148(7), 2207-2216 (1992).

38. Carter KC, Dolan TF, Alexander J, Baillie AJ, McColgan C. Visceral leishmaniasis: drug carrier system characteristics and the ability to clear parasites from the liver, spleen and bone marrow in *Leishmania donovani* infected BALB/c mice. *J Pharm Pharmacol*. 41(2), 87-91 (1989).

39. Collins M, Carter KC, Baillie AJ, O'Grady J. The distribution of free and non-ionic vesicular sodium stibogluconate in the dog. *J Drug Target*. 1(2), 133-142 (1993).

40. Mullen AB, Baillie AJ, Carter KC. Visceral leishmaniasis in the BALB/c mouse: a comparison of the efficacy of a nonionic surfactant formulation of sodium stibogluconate with those of three proprietary formulations of amphotericin B. *Antimicrob Agents Chemother*. 42(10), 2722-2725 (1998).

41. Williams D, Mullen AB, Baillie AJ, Carter KC.Comparison of the efficacy of free and non-ionic-surfactant vesicular formulations of paromomycin in a murine model of visceral leishmaniasis. *J Pharm Pharmacol*. 50(12), 1351-1356 (1998).

42. Kansal S, Tandon R, Dwivedi P *et al*. Development of nanocapsules bearing doxorubicin for macrophage targeting through the phosphatidylserine ligand: a system for intervention in visceral leishmaniasis. *J Antimicrob Chemother*. 67(11), 2650-2660 (2012).

43. Immordino ML, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. *Int J Nanomedicine*. 1(3), 297-315 (2006).

44. Daftarian PM, Stone GW, Kovalski L *et al*. A targeted and adjuvanted nanocarrier lowers the effective dose of liposomal amphotericin B and enhances adaptive immunity in murine cutaneous leishmaniasis. *J Infect Dis*. 208(11), 1914-1922 (2013).

45. Khan N, Gowthaman U, Pahari S, Agrewala JN. Manipulation of costimulatory molecules by intracellular pathogens: veni, vidi, vici!! *PLoS Pathog*. 8(6), e1002676 (2012).

46. Peine KJ, Gupta G, Brackman DJ, Papenfuss TL, Ainslie KM, Satoskar AR, Bachelder EM. Liposomal resiquimod for the treatment of Leishmania donovani infection. J *Antimicrob Chemother*. (2013)

47. Das A, Ali N.Vaccine Development Against *Leishmania donovani*. *Front Immunol*. 15 (99), (2012).

48. Nakano M, Fukuda M, Kudo T, Miyazaki M, Wada Y, Matsuzaki N, Endo H, Handa T. Static and dynamic properties of phospholipid bilayer nanodiscs. *J Am Chem Soc*. 131(23), 8308-8312 (2009).

49. Tufteland M, Ren G, Ryan RO. Nanodisks derived from amphotericin B lipid complex. *J Pharm Sci*. 97(10), 4425-4432 (2008).

50. Nelson KG, Bishop JV, Ryan RO, Titus R. Nanodisk-associated amphotericin B clears *Leishmania major* cutaneous infection in susceptible BALB/c mice. *Antimicrob Agents Chemother*. 50(4), 1238-1244 (2006).

51. Nguyen TS, Weers PMM, Raussens V *et al.* Amphotericin B induces interdigitation of apolipoprotein stabilized nanodisk bilayers. *Biochim Biophys Acta*. 1778(1), 303–312 (2008).

52. Lemke A, Kiderlen AF, Kayser O. Amphotericin B. *Appl. Microbiol. Biotechnol*. 68, 151–162 (2005).

53. Gaspar R, Opperdoes FR, Préat V, Roland M. Drug targeting with polyalkylcyanoacrylate nanoparticles: in vitro activity of primaquine-loaded nanoparticles against intracellular Leishmania donovani. *Ann Trop Med Parasitol.* 86(1), 41-49 (1992).

54. Paul M, Durand R, Boulard Y *et al*. Physicochemical characteristics of pentamidine-loaded polymethacrylate nanoparticles: implication in the intracellular drug release in *Leishmania major* infected mice. *J. Drug Target*. 5, 481–490 (1998).

55. Nahar M, Jain NK. Preparation, characterization and evaluation of targeting potential of amphotericin B-loaded engineered PLGA nanoparticles. *Pharm Res*. 26(12), 2588-2598 (2009)

56. Van de Ven H, Vermeersch M, Vandenbroucke RE *et al*. Intracellular drug delivery in Leishmania-infected macrophages: Evaluation of saponin-loaded PLGA nanoparticles. *J Drug Target*. 20(2), 142-154 (2011).

57. Van de Ven H, Paulussen C, Feijens PB *et al*. PLGA nanoparticles and nanosuspensions with amphotericin B: Potent *in vitro* and *in vivo* alternatives to Fungizone and AmBisome. *J Control Release.* 161(3), 795-803 (2012).

58. Li H, Yu Y, Faraji Dana S, Li B, Lee CY, Kang L. Novel engineered systems for oral, mucosal and transdermal drug delivery. *J Drug Target*. 21(7), 611-629 (2013).

59. Diab R, Jaafar-Maalej C, Fessi H, Maincent P. Engineered nanoparticulate drug delivery systems: the next frontier for oral administration? *AAPS J*. 14(4), 688-702 (2012).

60. Bhandari V, Kulshrestha A, Deep DK. Drug susceptibility in *Leishmania* isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. *PLoS Negl Trop Dis*. 6(5) (2012).

61. Rijal S, Ostyn B, Uranw S. Increasing failure of miltefosine in the treatment of kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or noncompliance. *Clin Infect Dis*. 56(11), 1530-1538 (2013).

62. Cavalla D. Predictive methods in drug repurposing: gold mine or just a bigger haystack? *Drug Discov Today*. 18(11-12), 523-532 (2013).

63. Mazzaferro S, Bouchemal K, Ponchel G. Oral delivery of anticancer drugs III: formulation using drug delivery systems. *Drug Discov Today.* 18(1-2), 99-104 (2013).

64. Prajapati VK, Awasthi K, Yadav TP, Rai M, Srivastava ON, Sundar S. An oral formulation of amphotericin B attached to functionalized carbon nanotubes is an effective treatment for experimental visceral leishmaniasis. *J Infect Dis*. 205(2), 333-336 (2012).

65. Prajapati VK, Awasthi K, Gautam S, Yadav TP, Rai M, Srivastava ON, Sundar S. Targeted killing of *Leishmania donovani in vivo* and *in vitro* with amphotericin B attached to functionalized carbon nanotubes. *J Antimicrob Chemother*. 66(4), 874-879 (2011).

66. Coyuco JC, Liu Y, Tan BJ, Chiu GNC. Functionalized carbon nanomaterials: exploring the interactions with Caco-2 cells for potential oral drug delivery. *Int J Nanomedicine* (6), 2253-2263 (2011).

67. Shvedova A.A., Castranova V, Kisin E.R., Schwegler-Berry D, Murray A.R., Gandelsman, V.Z. Maynard A., Baron P. Exposure to carbon nanotube material: assessment of nanotube cytotoxicity using human keratinocyte cells. *J. Toxicol. Environ. Health*. 66: 1909–1926 (2003).

68. Veerareddy PR Vobalaboina V, Nahid A. Formulation and evaluation of oil-in-water emulsions of piperine in visceral leishmaniasis. *Pharmazie*. 59(3), 194-197 (2004).

69. Wasan EK, Gershkovich P, Zhao J *et al*. A novel tropically stable oral amphotericin B formulation (iCo-010) exhibits efficacy against visceral Leishmaniasis in a murine model. *PLoS Negl Trop Dis*. 4(12), e913 (2010).

70. Italia JL, Kumar MN, Carter KC. Evaluating the potential of polyester nanoparticles for per oral delivery of amphotericin B in treating visceral leishmaniasis. *J Biomed Nanotechnol*. 8(4), 695-702 (2012).

71. Rubin BK. Air and soul: the science and application of aerosol therapy. *Respir Care*. 55(7), 911-21 (2010).

72. Darquenne C. Aerosol deposition in health and disease. *J Aerosol Med Pulm Drug Deliv*. 25(3), 140-147 (2012).

73. Zarogoulidis P, Chatzaki E, Porpodis K, *et al*. Inhaled chemotherapy in lung cancer: future concept of nanomedicine. *Int J Nanomedicine*. 7, 1551-72 (2012).

74. Westphalen K, Gusarova GA, Islam MN, Subramanian M, Cohen TS, Prince AS, Bhattacharya J.Sessile alveolar macrophages communicate with alveolar epithelium to modulate immunity. *Nature*. 506(7489), 503-506 (2014).

75. GeurtsvanKessel1 CH, B N Lambrecht B.N. Division of labor between dendritic cell subsets of the lung. *Mucosal Immunology*. 1, 442–450 (2008).

76. Alsaadi M, Italia JL, Mullen AB *et* al. The efficacy of aerosol treatment with non-ionic surfactant vesicles containing amphotericin B in rodent models of leishmaniasis and pulmonary aspergillosis infection. *J Control Release*. 160(3), 685-691 (2012).

77. Elsabahy M, Wooley KL. Design of polymeric nanoparticles for biomedical delivery applications. *Chem Soc Rev*. 41(7), 2545-2561 (2012).

78. El-On J, Jacobs GP, Witztum E, Greenblatt CL. Development of topical treatment for cutaneous leishmaniasis caused by *Leishmania major* in experimental animals. *Antimicrob Agents Chemother*. 26(5), 745-51 (1984).

79. Carneiro G, Santos DC, Oliveira MC *et al*. Topical delivery and *in vivo* antileishmanial activity of paromomycin-loaded liposomes for treatment of cutaneous leishmaniasis. *J Liposome Res*. 20(1), 16-23 (2010).

80. Jaafari MR, Bavarsad N, Bazzaz BS *et al*. Effect of topical liposomes containing paromomycin sulfate in the course of Leishmania major infection in susceptible BALB/c mice. *Antimicrob Agents Chemother*. 53(6), 2259-2265 (2009)

81. Berning CK, Griffith JF, Wild JE. Research on the effectiveness of denatonium benzoate as a deterrent to liquid detergent ingestion by children. *Fundam Appl Toxicol*. 2(1), 44-48 (1982).

82. Thalhofer CJ, Graff JW, Love-Homan L *et al*. *In vivo* imaging of transgenic *Leishmania* parasites in a live host. *J Vis Exp*. 27;(41), (2010).

83. Brogan J, Li F, Li W, He Z, Huang Q, Li CY. Imaging molecular pathways: reporter genes. *Radiat Res*. 177(4), 508-13 (2012).

84. Bringmann G, Thomale K, Bischof S *et al*. A novel *Leishmania major* amastigote assay in 96-well format for rapid drug screening and its use for discovery and evaluation of a new class of leishmanicidal quinolinium salts. *Antimicrob Agents Chemother*. 57(7), 3003-3011 (2013).

85. Bhandari V, Sundar S, Dujardin JC, Salotra P. Elucidation of cellular mechanisms involved in experimental paromomycin resistance in *Leishmania donovani*. *Antimicrob Agents Chemother*. 2014 (in press).

86. Mukherjee A, Padmanabhan PK, Singh S, *et al.* R. Role of ABC transporter MRPA, gamma-glutamylcysteine synthetase and ornithine decarboxylase in natural antimony-resistant isolates of Leishmania donovani. *J Antimicrob Chemother*. 59(2), 204-11 (2007).

87. Zinzi L, Capparelli E, Cantore M, Contino M, Leopoldo M, Colabufo NA. Small and Innovative Molecules as New Strategy to Revert MDR. *Front Oncol*. 4:2, (2014).

Fig legends

Fig. 1 The route a drug must take to access intracellular *Leishmania* amastigotes within macrophages. A drug enters the body by the route administered and has to reach the sites where infected macrophages reside and as a consequence have to cross multiple membranes to enter the parasite (N is the nucleus of the cell).

Fig. 2. Imaging of mice and organs samples using the IVIS® inaging system. Mice (A and B) were inoculated intravenously with 5 x 10^5 B16 F0 luc cells and 30 minutes later treated intravenously with luciferin solution (free, 3.38 mg/ml; 30 mg/kg)) or luciferin-NIV (NIV, prepared using luciferin solution at 18.69 mg/ml and diluted 1:5 just before use; 30 mg/kg). Animals were imaged at 2 min intervals from 10 minutes after dosing. The results for 14 (A) and 28 (B) minutes after dosing clearly show that using NIV improves delivery of luciferin to the cancer cells. BALB/c mice were inoculated with 2 x 10^7 luciferase expressing *L. donovani* and treated intravenously with luciferin solution (Free) or luciferin-NIV (NIV) using the protocol for A and B. 30 minutes after treatment the mice were sacrificed and the liver and spleen of each animal removed. The organs were imaged immediately after removal (C) or after immersion in luciferin solution (D, 150 µg/ml PBS pH 7.4) for 5 mins to allow comparison of the total parasite burdens present (D) in the spleen and liver and the parasite burdens exhibited by *in vivo* treatment with the two luciferin formulations. The results clearly show that using NIV improved delivery of luciferin to the parasites and maintained luciferin level longer than that achieved for luciferin solution. Detailed methods are given in Alsaadi *et al.* [*52*].