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Route map for the discovery and pre-clinical development of new drugs and treatments for cutaneous leishmaniasis

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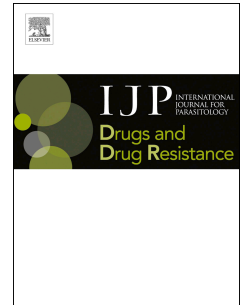
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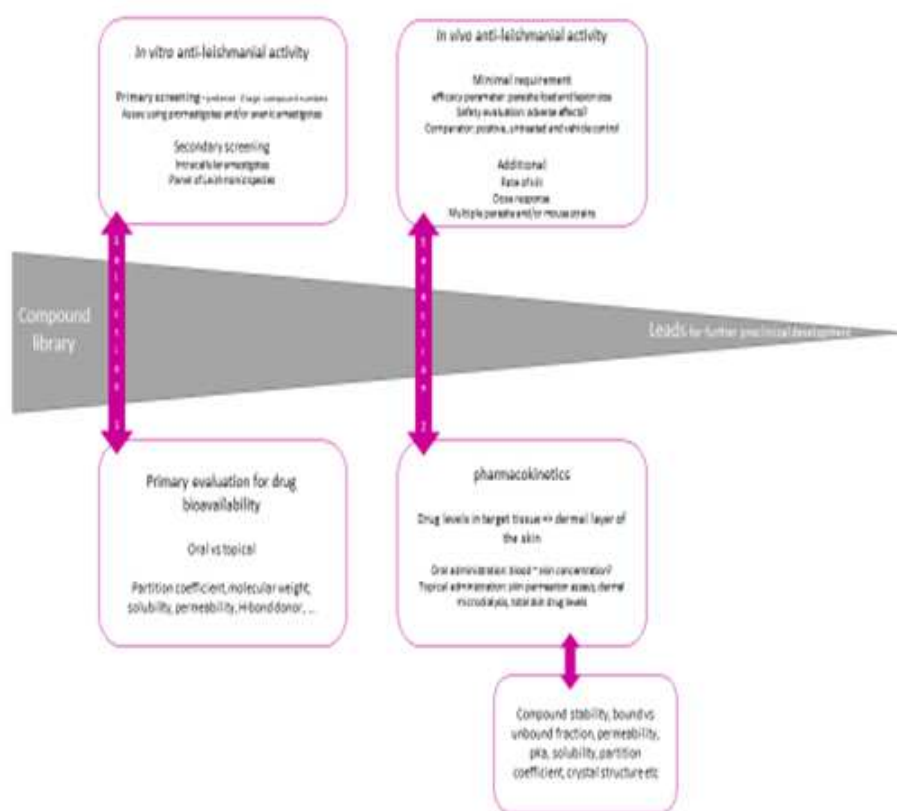
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## Graphical Abstract



## Route map for the discovery and pre-clinical development of new drugs and treatments for Cutaneous Leishmaniasis

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### Keywords

Cutaneous *Leishmaniasis*, drug discovery, drug development, *in vitro* assays, *in vivo* models, pharmacokinetics, formulations, immunomodulatory drugs

### Abstract

Although there have been significant advances in the treatment of visceral leishmaniasis (VL) and several novel compounds are currently in pre-clinical and clinical development for this manifestation of leishmaniasis, there have been limited advances in drug research and development (R & D) for cutaneous *leishmaniasis* (CL). Here we review the need for new treatments for CL, describe *in vitro* and *in vivo* assays, models and approaches taken over the past decade to establish a pathway for the discovery, and pre-clinical development of new drugs for CL. These recent advances include novel mouse models of infection using bioluminescent *Leishmania*, the introduction of PK/PD approaches to skin infection, and defined pre-clinical candidate profiles.

**Abbreviations:** Cutaneous leishmaniasis – CL; Mucocutaneous leishmaniasis – MCL; Visceral leishmaniasis – VL; Pharmacokinetics PK; target product profile - TPP; target compound profile - TCP; diffuse cutaneous Leishmaniasis - DCL; disseminated Leishmaniasis – DL; Drugs for Neglected Diseases initiative – DNDi; area under the curve - AUC; no observed adverse effect - NOAEL;

minimum effective dose - MED; oral - PO; intra muscular - IM; adverse reactions - AR; antimicrobial peptides - AMP; oligodeocynucleotides - ODN; research and development - R & D.

## Introduction – Cutaneous Leishmaniasis

Cutaneous leishmaniasis (CL) is a neglected disease with an estimated global incidence of 600,000-1,000,000 new cases every year, which mainly affects children in poor areas (Alvar et al., 2012). Unlike VL, CL is not life threatening, although it causes disfiguring lesions mostly on exposed body parts such as the face, arms, and legs. Hence, affected people are stigmatized and subjected to ostracism and, have impaired access to education, marriage, and well-paid jobs, which leads to economic losses and psychological damage (Bennis et al., 2018), (Bailey et al., 2019).

According to the World Health Organization (WHO), CL is endemic in 87 countries around the world, predominantly in Afghanistan, Pakistan, the Islamic Republic of Iran, Saudi Arabia, the Syrian Arab Republic, Algeria, Ethiopia, Brazil, Colombia, and Peru. The highest proportion of affected countries is found in the Eastern Mediterranean region, which suffers political instability, wars and migrant movements; followed by the Region of the Americas. Some countries such as Belize, Thailand, and the United States have been recently included in the list of endemic territories' list ("Global Leishmaniasis update, 2006–2015: a turning point in Leishmaniasis surveillance.," 2017).

CL displays several clinical presentations depending on the *Leishmania* species, host immunity, and transmission. A typical lesion of CL is a painless papule or ulcer at the site where the female sand fly feeds. In a variable proportion of cases, it may self-cure within 3-18 months, often developing into an ulcer covered with an adherent crust of dried exudate during this period. In the Old World countries, CL is mostly caused by *L. major*, *L. aethiopica* (zoonotic transmission), and *L. tropica* (anthroponotic transmission). All species are able to produce multiple lesions that tend to heal slowly and leave large and disfiguring scars. *L. aethiopica* can also metastasize to oronasal regions or across the whole body skin as diffuse CL. *L. infantum* usually causes generally single nodular lesions that heal slowly but may also affect the oronasal region (Burza et al., 2018).

In the New World countries, primary lesions may be single or multiple, but lymphadenitis and lymphadenopathy are common to lesions caused by species of the *Viannia* subgenus. In addition, secondary cutaneous and mucosal lesions may occur. Parasites spread to the mucous membranes, especially those of the nose, mouth, and throat, and cause extensive damage and disfiguration, thus giving rise to mucocutaneous Leishmaniasis (MCL). This condition is mainly caused by *L. braziliensis* and *L. panamensis/guyanensis* (all species of the subgenus *Viannia*), although it can result from infection by other species (Amato et al., 2007). Disseminated Leishmaniasis (DL) is another clinical presentation mostly associated with *L. braziliensis* infections where tens to thousands of polymorphic tegumentary lesions develop on several areas of the body (Machado et al., 2019). Parasites are present in small numbers in lesions with signs of a strong inflammatory response (Machado et al., 2019). In the New World, another rare but severe form of tegumentary disease is diffuse cutaneous Leishmaniasis (DCL) associated mainly with infections by *L. amazonensis*. DCL is anergic, characterized by multiple infiltrated plaques and nodules, and an absence of ulcers or mucosal involvement. In addition, DCL is characterized by uncontrolled proliferation of parasites in macrophages and an absence of immune infiltration in the lesions (Convit et al., 1972). Cure rates for simple CL, without treatment, are dependent on the species; for example, 44% - 72% for *L. mexicana* and 6.2% - 20% for *L. braziliensis* (Cota et al., 2016).

## Cutaneous Leishmaniasis – Current Drugs and Treatments

Current treatments for CL have been poorly justified through clinical trials and have sub-optimal effectiveness (Gonzalez et al., 2008), (Gonzalez et al., 2009). Treatment has long depended on antiquated drugs that are considered far too toxic for introduction under modern registration systems. None of the current systemic therapies, including pentavalent antimonials, miltefosine, and amphotericin B used for CL, were conceived or developed as specific treatments for CL. If the drug works for VL, then it is tested and adapted for its use in CL populations, despite the fact that the pharmacokinetic properties of the drug used to treat a patient with VL are often different to those for patients with CL. For example, liposomal amphotericin B, which is effective in most foci of VL (except East Africa), displays variable cure rates in CL (Solomon et al., 2013), (Guery et al., 2017).

WHO treatment recommendations for CL are based on the causative species, geographical area, and the clinical features of the disease. Recommendations vary from no treatment, mainly for infections due to *L. mexicana* or *L. major*, to topical or systemic approaches. Local therapies, including thermotherapy or cryotherapy with or without local infiltration with antimonials, as well as paromomycin ointment, are options with less systemic toxicity but variable efficacy. These methods are recommended for subjects with infections due to *L. mexicana* or *L. major*, or for patients with small and few lesions. Systemic treatments on the other hand, such as miltefosine, pentavalent antimonials, pentamidine, or amphotericin B formulations, are recommended for more complicated cases, for non-responders to topical treatments, immunosuppressed patients, and for areas where progression to mucosal leishmaniasis is prevalent. Miltefosine (Impavido®) was registered with the FDA in 2014 for CL infections due to *L. braziliensis*, *L. panamensis*, and *L. guyanensis*. There is not much evidence of miltefosine efficacy against infections caused by Old World species (van Thiel et al., 2010), (Lee and Hasbun, 2003), (Velez et al., 2010), (Copeland and Aronson, 2015). Current treatments for CL have been reviewed in depth in two recent publications (Aronson, 2017), (Burza et al., 2018). Here we focus on the pathway required to discover and develop new treatments for this disease.

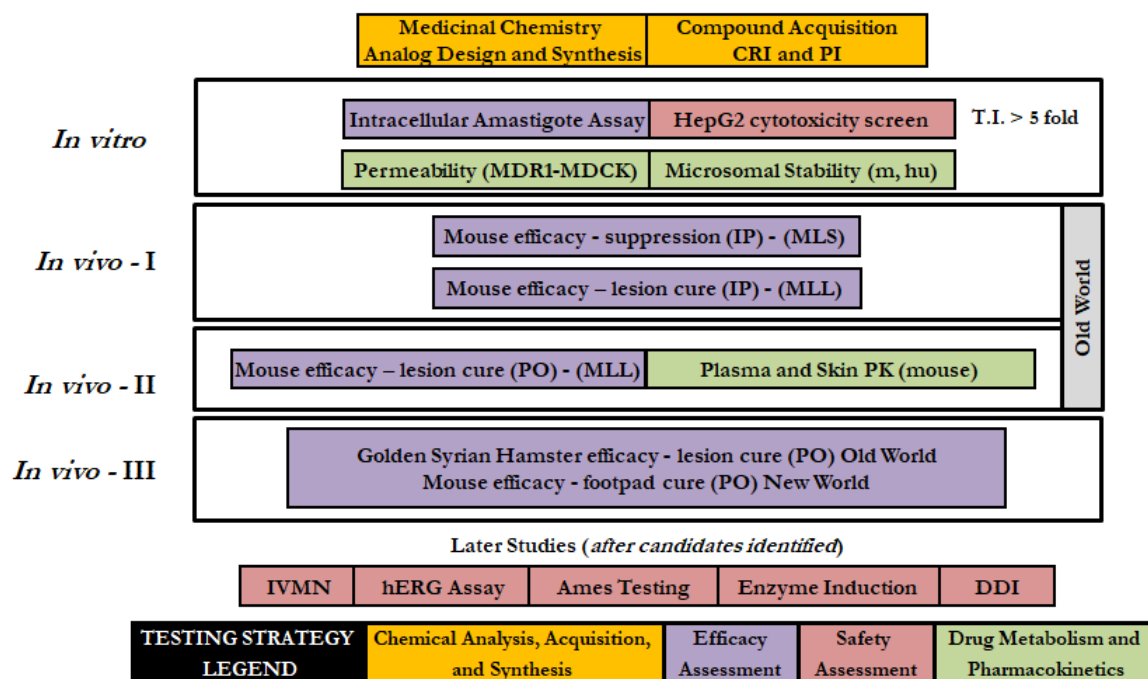
### **Cutaneous Leishmaniasis Research Directions, Gaps, and Needs for New Drugs and Treatments**

Over the past decade, there has been significant progress in the treatment and pre-clinical development of drugs for VL (Alves et al., 2018), (Van den Kerkhof et al., 2018). In contrast, R & D for new drugs and treatments for CL were left behind. There is no coherent “end-to-end” strategy even though there have been several new approaches, re-iterations of old ones, and the recent identification of new potential drugs and formulations. The one part of the CL R & D pathway that has received significant attention is clinical trials and consequently, clear progress has been made. Reviews of clinical studies undertaken on drugs and treatments for CL showed the inadequacy of most of these studies and the need for a re-consideration of the design and analysis of clinical trials, including endpoints (Gonzalez et al., 2008), (Gonzalez et al., 2009). This problem was addressed and there is now a clinical methodology to assess new drugs and treatments (Gonzalez et al., 2010), (Olliario et al., 2013), (Olliario et al., 2018).

Having established the clinical part of the CL drug development pathway, the essential questions for this review are: (i) - do we have any optimised lead compounds, novel chemical entities, or re-positioned drugs, novel formulations, or adapted treatments to test in clinical trials? and (ii) if we do, is there an agreed and tested pre-clinical pathway that will effectively advance active compounds to candidate drugs? In this review, which in part follows a workshop held at the WorldLeish 6 Congress, May 2017, (<http://worldleish2017.org/#/>), we will focus on the potential routes to take new drugs and treatments from discovery to clinical trials. We will examine the relevance and use of assays and models for evaluation, the value of PK/PD analysis, systemic and

topical formulations, and roles for immunomodulatory compound combinations, and re-purposing of drugs. Some of these have previously been presented by the Walter Reed Army Institute of Research (WRAIR) in a validated, cost effective, gated-tier strategy as a possible approach to CL drug discovery using *in vitro* assays to identify hits which progress to more clinically relevant *in vivo* models (Grogl et al., 2013), (Caridha et al., 2017).

## Cutaneous Leishmaniasis Testing Strategy



**Figure 1: WRAIR Cutaneous Leishmaniasis Testing Strategy.** Analogs, designed or acquired, are assessed for activity (intracellular amastigote) and potential cytotoxicity. All analogs meeting cutoff criteria (assigned per case) but with a therapeutic index > 5 are tested for microsomal stability in mouse and human microsomes. These higher throughput assays gate the more costly, clinically relevant, animal models. *In vivo* efficacy in mice is initially tested against Old World (*L. major*) parasites, first in a lesion suppression model, then if warranted advanced to the rigorous lesion cure model. Initial *in vivo* experiments use intraperitoneal (IP) route of administration to maximize chance of success and provide early dose ranging. If successful, skin and plasma mouse PK is performed followed by oral (PO) dosing in the mouse lesion cure model (supporting our TPP). The final tier of *in vivo* efficacy assesses activity via PO dosing in a second animal species (GSH) as required for FDA approval and efficacy against a New World strain of *Leishmaniasis* in a mouse footpad model. Once late-stage candidates are identified early safety, assessment is performed as outlined above.

### Product Characteristic Profiles for New Cutaneous Leishmaniasis Treatments

What sort of drug or treatment do we need and what is the decision pathway for development? A clear set of indicators are needed to guide the CL drug R & D process and to ensure decision-making points are identified and routes/options for progress defined. These are normally outlined in a Target Product Profile (TPP), which is a planning tool that describes the desired R&D outcome and enables selection, progression, and management according to well-defined decision matrices. In

addition, further parameters are outlined in a pre-clinical Target Candidate Profile (TCP), which is a description of the physicochemical, anti-leishmanial, pharmacokinetic, safety, and formulation properties of a compound selected for pre-clinical development as a prelude to clinical studies with the potential to ultimately meet the TPP. The Drugs for Neglected Diseases initiative has established a TPP and TCP for CL which are shown in Table 1 and 2 (<https://www.dndi.org/diseases-projects/Leishmaniasis/tpp-cl/>).

Table 1: Target Product Profile for New CL Treatments

ATTRIBUTE	TARGET (IDEAL)	MINIMALLY ACCEPTABLE
Target Species	All <i>Leishmania</i> species	<i>L. tropica</i> or <i>L. braziliensis</i>
Safety monitoring requirement	None	Peripheral Health Centre. No major safety concerns.
Target population	Immunocompetent and immunosuppressed	Immunocompetent
Age / Gender	No restrictions	> 9 months of age
Use in pregnancy	Yes	No
Tolerability	Well tolerated. All AR's $\leq$ grade 1	Systemic AR grade 2-3 in <5%. Local AR $\leq$ grade 2-3 in <30%. No Treatment associated mortality
Contraindications	None	Females and males of reproductive potential
Efficacy (3M)	>95% patients	60% for <i>L. tropica</i> , 70% for <i>L. braziliensis</i>
Route of administration	Topical / oral	Non-parenteral, or few doses, if parenteral
Topical	$\leq$ 14 days	28 days
Oral	$\leq$ 7 days	28 days
Parenteral	No	3 injections
Stability	No cold chain, at least 3 years at 37°C	2 years at 4-8°C
Cost per treatment	To be defined	To be defined

Table 2: Target Candidate Profile (TCP) for New Pre-clinical Candidates for CL Administered by Systemic Administration

ATTRIBUTE	ACCEPTABLE (OLD WORLD <u>OR</u> NEW WORLD)	IDEAL (OLD WORLD <u>AND</u> NEW WORLD)
Efficacy	<p><i>In vivo</i>: &gt;95% reduction in lesion size in mouse model with <i>L. tropica</i> <b>OR</b> <i>L. braziliensis</i> (or the closest possible representative)</p> <p><i>In vitro</i>: Consistent activity within 10x vs. a panel of strains and isolates of <i>L. tropica</i> <b>OR</b> <i>L. braziliensis</i></p> <p><i>In vitro</i>: <math>E_{\max} &gt; 99\%</math><sup>1</sup></p>	<p>&gt;95% reduction in lesion size in mouse model with <i>L. tropica</i> <b>AND</b> <i>L. braziliensis</i> (or the closest possible representative)</p> <p>Consistent activity within 10x vs. a panel of strains and isolates from both New and Old World</p> <p><i>In vitro</i>: <math>E_{\max} &gt; 99\%</math></p>
Safety	<p><i>In vitro</i>: No <i>in vitro</i> signals preventing development<sup>2</sup></p> <p><i>In vivo</i> TI: <math>(\text{AUC at NOAEL}^3)/(\text{AUC at MED}_{95}^4) &gt; 10^5</math></p>	<p>No <i>in vitro</i> signals preventing development</p> <p><math>(\text{AUC at NOAEL})/(\text{AUC at MED}_{95}) &gt; 10</math></p>
CMC	Synthesis and formulation acceptable to enable PO or IM dosing	Synthesis and formulation acceptable to enable PO or IM dosing
DMPK	<p>Oral dosing: Human dose prediction &lt; 30mg/kg/day given QD or BID for a maximum of 28 days</p> <p>Parenteral dosing: A maximum of 3 injections over 7 days</p>	<p>Human dose prediction &lt; 30mg/kg/day given QD or BID for a maximum of 7 days</p> <p>A maximum of 3 injections over 7 days</p>

## Explanatory notes:

<sup>1</sup> Compound able to give *in vitro* >99% reduction of intracellular amastigotes relative to untreated control

<sup>2</sup> Includes: mammalian cytotoxicity, HERG, Ames, micronucleus, broad profiling

<sup>3</sup> Determined in rat repeat dose toxicology for duration  $\geq$  length of treatment in efficacy model

<sup>4</sup> Minimum dose required to achieve >95% reduction in lesion size *in vivo*

<sup>5</sup> Applies equally to **both** total AUC and free AUC comparisons.

These documents are not “set-in-stone” and can be modified through evidence and relevant discussion. A second point from the TPPs, as outlined by DNDi, is limiting the minimal acceptable criteria to “effectiveness against *L. tropica* and/or *L. braziliensis* alone” as they are considered to be the most difficult to treat. CL is a complex of clinical manifestations with different host-parasite interactions (including immune responses) for most species (Scott and Novais, 2016). Whether there should be species specific limitations should be considered with respect to three points:

- It may be a challenge to find a drug/treatment that works against all forms of CL.



- It will be very difficult to afford or attract major partners to develop multiple drugs for CL since it has been very difficult to develop one, namely the paromomycin topical formulation (Ben Salah et al., 2013), (Grogl et al., 1999).
- Given the areas of potential use of these species-specific treatments, companion diagnostics will also be needed.

### ***In vitro* and *In vivo* Models for Drug Development**

Drug development against the *Leishmania* parasite, a eukaryote organism that survives and divides in a low pH inside of a macrophage vacuole in different tissues in the body, can be difficult. A successful drug needs to either kill the parasite in the macrophage or activate the macrophage to do the job; whichever, the compound must cross up to 7 membranes and pH changes to reach the amastigote with implications for both compound pharmacokinetics and drug targeting (Lamotte et al., 2017, Croft, 2017). Although there has been progress in the development and use of *in vitro* assays and screens, there is no perfect animal model for *in vivo* testing of potential antileishmanial compounds (Figures 1 and 2). These limitations have contributed to the lack of novel compounds that have progressed into clinical development.

#### **(I) *In vitro* Assays**

To identify novel chemical entities with activity against those species of *Leishmania* that cause CL, *in vitro* screens should be based upon assays that: (i) provide active or dividing populations of the dividing amastigote, (ii) a measure of drug activity that is readily quantified, both kill and preferably rate of kill, (iii) accurately show the activity of standard drugs (ideally two standards per assay) at concentrations close to those achievable in serum or tissues (over a long time-course if necessary), (iv) use a panel of clinical isolates, as there is known strain/species variation in drug susceptibility, (v) ensure that these clinical isolates are of recent origin as there is a defined genetic drift in isolates maintained in culture over a long period, and (vi) ensure host cell factors, for example type of macrophage used are considered in analysis (Croft et al., 2006), (Bussotti et al., 2018), (Seifert et al., 2010). In the past, *in vitro* compound screening has also used promastigotes and axenic amastigotes. Promastigotes are grown in large quantities for large screening campaigns but targeting the insect stage is not predictive of activity against the clinically relevant amastigote stage. Temperature and pH can be altered to derive axenic amastigotes to screen compounds in quantities comparable to promastigote screens that demonstrate stage specific differences for compounds (Vermeersch et al., 2009). However, the development of imaging and high-content screening methods for *L. donovani* that has come with the advent of reporter transfected parasites, has made high throughput screening of intracellular parasites possible and has negated the advantage of axenic screens (De Rycker et al., 2013), (Tegazzini et al., 2016), (Siqueira-Neto et al., 2012). The difference for CL screening to that for VL is the need to test against multiple species. Due to the diversity and variability of *Leishmania* parasites, when using a standardized intracellular amastigote assay, lead drug candidates are screened against a panel of *Leishmania* species from geographically diverse areas. *In vitro* screening against multiple species from different geographic regions will not take into account diversity of the host, but can give EC<sub>50</sub> diversity data on a larger scale than could be achieved *in vivo*. For example, at WRAIR, potential antileishmanial compounds are tested *in vitro* against luciferase-expressing *L. amazonensis*, *L. major*, *L. mexicana*, *L. panamensis*, *L. guyanensis*, *L. peruviana*, and *L. tropica*. However, even using the best *in vitro* models, there is a large attrition rate of active compounds when moved forward to *in vivo* models. To make *in vitro* models more predictive, we need more physiologically relevant assays (Zulfiqar et al., 2017). With

the advent of organ on a chip technology, a 3D immunocompetent model of human skin is used to study drug efficacy on human cutaneous lesions (Horvath et al., 2016).

## (II) *In vivo* Models

Confirming the *in vivo* efficacy of potential antileishmanial compounds that display *in vitro* potency and good chemical characteristics, is one of the most important steps in pre-clinical research. Traditional *in vivo* lesion cure (dorsal and footpad) models using the inbred BALB/c mouse and Golden Syrian Hamster (GSH) infected with high inoculums of *Leishmania spp.* parasites as a source of CL infection, have been widely used for decades to test the efficacy of potential antileishmanial drugs (Mears et al., 2015), (Croft et al., 2006), (Robledo et al., 2012), (Milon et al., 1995), (Gomes-Silva et al., 2013), (Gamboa et al., 2008), (Ribeiro-Romao et al., 2014). The highly susceptible BALB/c mouse /*L. major* lesion cure model is highly reproducible, consistent, and has some clinical similarities with human CL. Nonetheless, this model is extremely stringent, not likely to detect all active antileishmanial compounds and has different pathological features and immunological responses compared to human CL (Mears et al., 2015). As a result, this model is less suitable for the definitive assessment of antileishmanial drug efficacy (Mears et al., 2015), (Croft et al., 2006). Presence of metastatic disease, which leads to death, is a major concern for using the BALB/c mouse/*Leishmania spp.* model in antileishmanial drug discovery (Loeuillet et al., 2016) (Figure 2). In general, lesion cure models are invasive, costly, and require a long incubation period before observations of drug efficacy can be made. As inflammation and secondary infections can contribute to the lesion size, accurate assessment of drug efficacy is also less consistent (Mears et al., 2015), (Croft et al., 2006). Use of biopsies with histopathology and polymerase chain reactions (PCR), scrapings, cytology brush, and lesion swabs coupled to qPCR offer high sensitivity and specificity and are a better indication of the degree of infection compared to using lesion cure as a sole experimental endpoint (Croft et al., 2006), (Adams et al., 2014), (Boggild et al., 2011). However, some of these methods are invasive and questions remain regarding what part of the lesion specimens should be collected from in order to accurately determine parasite load (Suarez et al., 2015).

Challenges remain for reproducible, predictive models of *L. tropica*, *L. aethiopica*, and *L. braziliensis*. *L. braziliensis* is particularly hard to mimic in animals since most *L. braziliensis* strains cause self-limited or asymptomatic infections in mice. Recently, an *L. braziliensis* strain was shown to lead to sustained localized disease in BALB/c mice, allowing for a more robust testing of drug efficacy (Coelho et al., 2016). However, no models of mucosal disease are available yet. Furthermore, models need to represent the diversity of immune response in *L. braziliensis* infections that play a fundamental role in the response to therapy as illustrated, for example, by the higher treatment failure rate when treatment is initiated very early in the course of the disease, before the cutaneous lesions ulcerate (Unger et al., 2009). It appears that early treatment leads to an enhanced production of pro-inflammatory cytokines that promote the development of ulcers in spite of antimony therapy. This adds another layer of complexity to translating animal model findings into clinical data (Costa et al., 2018).

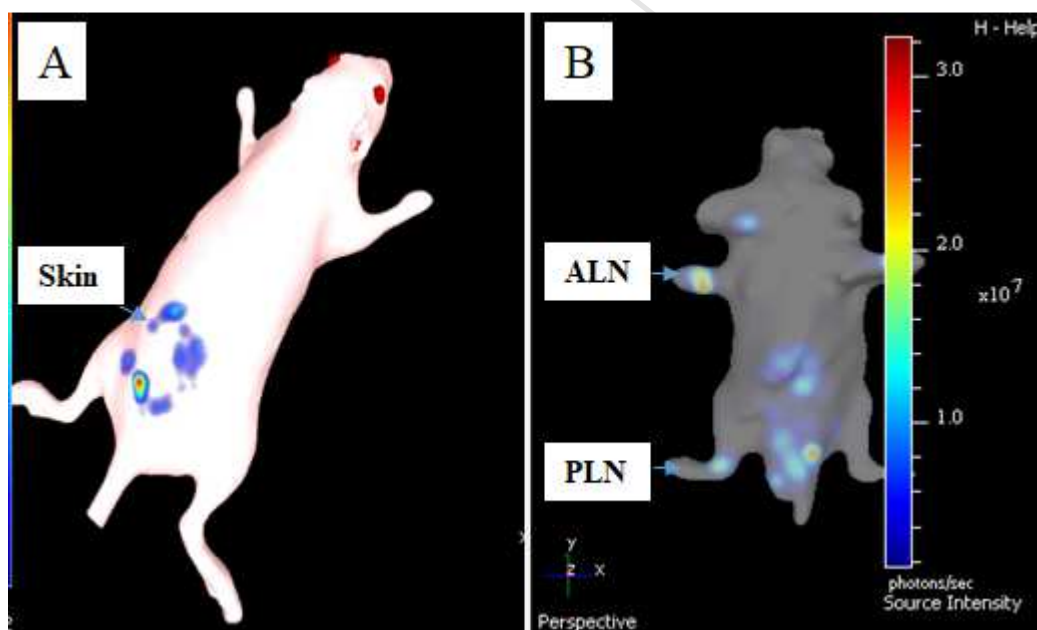
The most predictive animal models in antileishmanial drug discovery have been conducted in inbred mice that self-cure (such as C57BL/6, CBA, and CsS), GSH, and non-human primates (such as Syke's, vervet, baboons, and rhesus macaque monkeys). These models present similar clinical symptoms, histopathological features, and lesion cure patterns of human CL lesions that progress to ulceration and are followed by complete healing (Mears et al., 2015), (Gomes-Silva et al., 2013), (Loeuillet et al., 2016), (Githure et al., 1987), (Freidag et al., 2003), (Amaral et al., 2001), (Flynn et al., 2005), (Loria-Cervera and Andrade-Narvaez, 2014). Probst et al. have described a natural infection *L.*

*major*/Rhesus monkey model transmitted by *P. papatasi* sand-fly bites with high rates of infection and an antibody response similar to the human disease (Probst et al., 2001). Still, non-human primate models of leishmaniasis require large amounts of drugs for testing, present ethical problems, and as a result, should be used “under the most strict circumstances” as a third tier model for confirming antileishmanial drug efficacy before clinical trials begin (Mears et al., 2015). Obtaining consistent, non-variant infections in outbred rodent models can be very challenging, which makes their use expensive and unpractical (Mears et al., 2015). Females of two sand fly genera, *Phlebotomus* and *Lutzomyia*, respectively for the Old and New World leishmania parasites, are of medical importance as proven vectors of *Leishmania* species pathogenic for humans (Dostalova and Volf, 2012). Sand fly saliva is composed of secreted proteins, and in some cases, nucleosides and nucleic acids (Dostalova and Volf, 2012). Salivary proteins have a strong effect on the immune system of the host, which includes but is not limited to, inhibition of T cell and macrophage activation, diminishing the ability of dendritic cells to present antigens, and reducing neutrophil migration during specific antigen-induced inflammation (Abdeladhim et al., 2014). Therefore, whenever possible, animal infection should mimic the natural transmission by the sand-fly bite of a small inoculum of *Leishmania* metacyclic promastigotes and saliva in the infection site (Mears et al., 2015), (Croft et al., 2006), (Abdeladhim et al., 2014), (Kimblin et al., 2008), (Belkaid et al., 1998), (Belkaid et al., 2000), (Cawfield et al., 2018). Humanized mice models have also been used with some success to study the human immune response against the *Leishmania* pathogen, conduct drug discovery studies, and predict possible side effects of new drugs in humans (Wege et al., 2012).

During the past decade, use of noninvasive *in vivo* imaging technology has revolutionized pre-clinical antileishmanial drug discovery and development by providing a simple, accurate means to quantify the *Leishmania* parasite load in a live host (Mears et al., 2015), (Croft et al., 2006). Several probes such as the firefly luciferase reporter gene (LUC), the green and enhanced green fluorescent proteins (GFP and EGFP), mCherry red fluorescent protein (RFP), near-infrared fluorescent proteins (iRFP), or a combination of those have been used to monitor the *in vivo* intracellular proliferation of *Leishmania spp.* parasites (Dube et al., 2009, Gupta and Nishi, 2011), (Lecoeur et al., 2010), (Rocha et al., 2013), (Pulido et al., 2012), (Mehta et al., 2008), (Lecoeur et al., 2007), (Calvo-Alvarez et al., 2012), (Roy et al., 2000), (Millington et al., 2010), (Calvo-Alvarez et al., 2015), (Filonov et al., 2011), (Bolhassani et al., 2011), (Reimao et al., 2013), (Coelho et al., 2016). Due to the strong correlation between the parasite load and fluorescence emission or luciferase activity, it is possible to assess *Leishmania* disease progression and measure drug efficacy without the requirement for animal subject sacrifice, which is in accordance with the three R's (Replacement, Reduction, Refinement) of animal testing. Studies have shown that the use of bioluminescent parasites to quantify *Leishmania spp.* infection in the ear is a more accurate approach compared with the more traditional measurements of lesion diameter, volume, and thickness (Schuster et al., 2014). Most recently, Caridha et al. described a new, real time, higher throughput longitudinal *in vivo* imaging BALB/c mouse/luciferase-expressing *L. major* lesion suppression model that serves as a prescreen to measure anti-leishmanial drug efficacy in only 18 days. This model is fast, less costly, causes minimal animal pain and suffering, and maximizes the number of potential antileishmanial compounds that can be tested, which makes it a good first tier animal model to assess compound efficacy (Caridha et al., 2017) (Figure 1).

A drug discovery-testing paradigm of cost effective *in vitro* and *in vivo* assays and clinically relevant *in vivo* models has proven to be an effective method to screen larger compound collections. Furthermore, knowing that physicochemical properties of compounds play a critical role on pharmacokinetics as well as compound efficacy, medicinal chemistry studies should be conducted in parallel with the efficacy ones. In the gated-tiered strategy described by Caridha et al. and Grogl et

al, in order to maximize chances for efficacy, the validated *in vitro* hits are initially dosed intraperitoneally (IP) in the BALB/c mouse/*L. major* lesion suppression prescreen model as well as lesion cure model. During these initial mouse *in vivo* studies, plasma drug concentrations are assessed by drawing 10  $\mu$ L blood samples extracted from a tail nick (Rahavendran et al., 2012). Information gathered at this stage plays a critical role in compound design. Lead compounds are later confirmed for oral (PO) efficacy in a second tier BALB/c mouse *L. major* lesion cure model. At WRAIR, efforts are made to avoid, even temporarily, the disseminated disease which makes the BALB/c mouse/*Leishmania spp.* lesion cure model extremely rigorous and might result in the “inability to detect potentially useful compounds” (Mears et al., 2015), (Figure 2). Drug treatments in efficacy studies in the lesion cure BALB/c mice models start when lesion sizes are  $\leq 20$  mm<sup>2</sup> compared to previously, when you had to wait for the average lesion size to be 50 mm<sup>2</sup> (Caridha et al, 2017). At this stage, full mouse pharmacokinetic analysis is conducted on promising compounds to predict oral dosing for follow on studies (Caridha et al., 2017), (Grogl et al., 2013), (Figure 1). In the third tier, lead compounds are further tested in a GSH/ *L. major* lesion cure model (Figure 1). In addition, in the third tier models, antileishmanial drug efficacy is assessed against New World species such as *L. panamensis* and *L. guyanensis*. As mentioned above, testing for antileishmanial efficacy against other Old and New World species that cause CL such as *L. tropica* and *L. braziliensis* is recommended (Mears et al., 2015), (David and Craft, 2009). Of course, the aforementioned issues surrounding immune status, stringency, and relevance to human clinical disease merit further exploration in order to maximize the predictive value of pre-clinical *in vivo* testing strategies.



**Figure 2: Disseminated *Leishmania* Disease in BALB/c Mice.** Mice were infected at the base of the tail with  $1 \times 10^7$  stationary phase luciferase expressing *L. major* parasites and the images were obtained through three dimensional reconstruction of bioluminescence using DLIT in a BALB/c mouse at forty days (image A) and seventy days (image B) post-infection. The brighter, red and yellow areas shown in the image are photon intensity measurements representing greater parasite counts than the dimmer green-blue areas. Forty days post infections (image A) the *L. major* parasites are visible only in the infected skin at edge of the infection (lesion) site\*. At seventy days post infections (image B) the *L. major* parasites are visible in large quantities in both popliteal and axillary lymph nodes (PLN, ALN) and possibly in the internal organs.\* In our laboratory, using the *in vivo*

imaging technology, the smallest number of parasites that can be visualized in BALB/c mice's skin immediately after infection is  $1.5 \times 10^4$  luciferase-expressing *L. major* promastigotes. The limit of detection for amastigotes in the skin and internal organs has not been determined. Other methods can detect presence of parasites in the internal organs at a much earlier time during the disease progression.

### Pharmacokinetics and Pharmaceutics

As most candidate drugs currently in the development pipeline for CL were initially identified as part of drug screenings against visceral *Leishmania* species, several key issues, in addition to species variation, in drug susceptibility (*ibid*) need to be considered ([https://www.dndi.org/2018/media-centre/news-views-stories/news/leish\\_rdn\\_status\\_2018/](https://www.dndi.org/2018/media-centre/news-views-stories/news/leish_rdn_status_2018/), accessed on 4-11-2018):

- (i) Drug distribution: As the *Leishmania* amastigotes are situated in the phagolysosome of macrophages located in the dermal layer of the skin at the borders of the lesion close to the inflammatory cells, compounds with PK properties for accumulation in visceral organs, for example 8-aminoquinolines, might not be appropriate
- (ii) There are additional options to the systemic treatment required for VL, which include topical formulations and local immunotherapy
- (iii) The impact of pathology / immunopathology on drug permeation and distribution in the lesion.

To ensure adequate efficacy against CL, a candidate drug requires delivery at the site of infection with optimal exposure, both concentration and time. A challenge for CL drug R & D is the integration of requisite PK and PD parameters into the selection of leads following compound screening and testing *in vivo* models as soon as possible (Figure 3, Table 2). The Lipinski and Dermal Rule of five describe certain physicochemical properties that are believed to govern drug bioavailability upon oral administration and skin penetration, respectively (Lipinski et al., 2001), (Naik et al., 2000). These properties include partition coefficient, molecular weight and H-bond donors, and are directly and/or indirectly involved in the administration, distribution, metabolism, and excretion processes that play a pivotal role in defining the disposition of a drug. The properties can be estimated using software and/or established using an experimental set up. At this stage, they should be considered a guideline rather than a go/no-go parameter (McKerrow and Lipinski, 2017). Here, we focus on the pharmacokinetics and pharmaceutics of topical drugs, which was the focus of the workshop held at the WorldLeish 6 Congress (<http://worldleish2017.org/#/>).



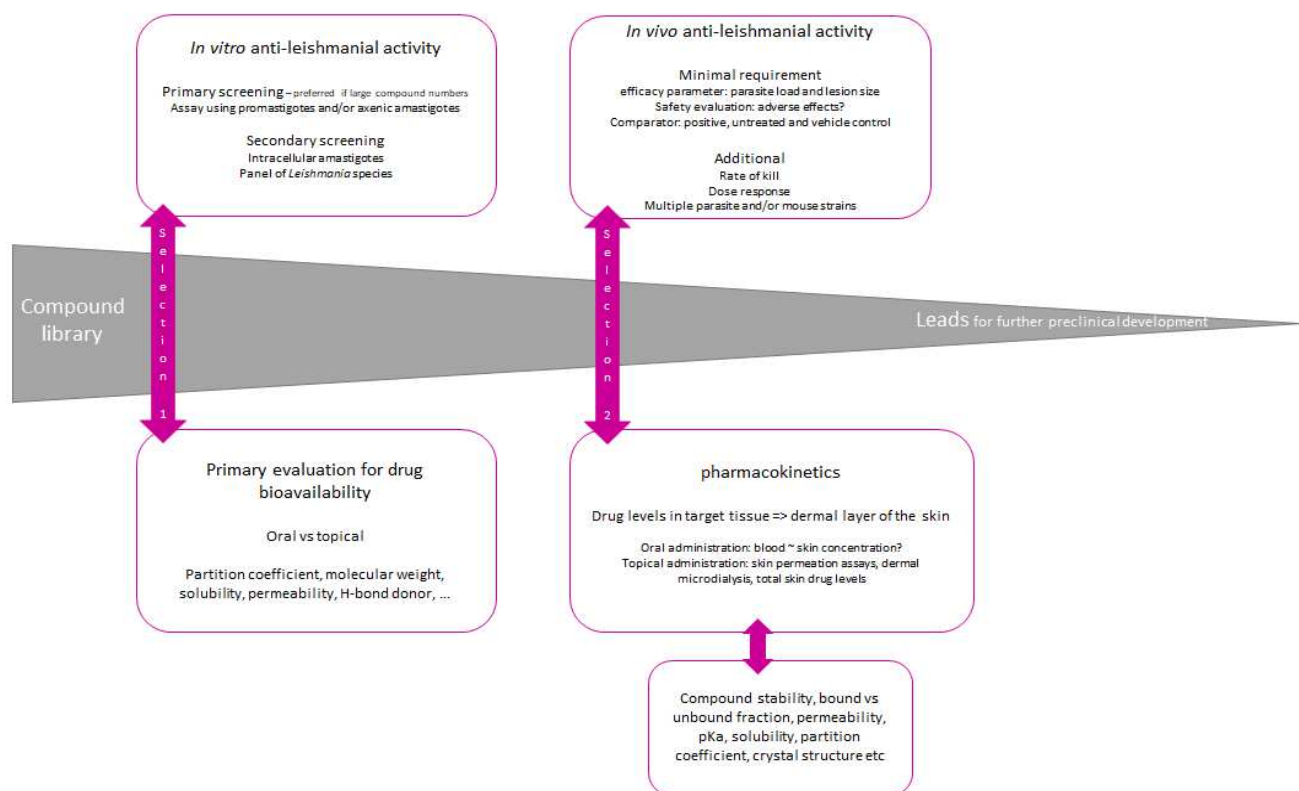


Figure 3. A Drug discovery progression pathway for CL including the evaluation of both pharmacokinetic and pharmacodynamic parameters.

Topical therapy is warranted for management of patients with few, localized, and uncomplicated lesion caused by Old and New World CL (WHO, Technical Report Series 949, 2010), (Blum et al., 2014), (Aronson, 2017). The skin is typically a peripheral compartment for drug distribution that can be reached either through systemic drug exposure whereby the drug is taken up in the blood stream and carried to the parasite-containing lesion skin via skin capillaries or, topically via penetration into the skin and lesion by a series of partitioning and diffusion steps. In the ideal scenario where CL lesions are diagnosed early and treated during the papule or nodular stage, a potent topical antileishmanial drug would show efficacy by penetrating all skin layers, targeting only the parasite-containing parts of the lesion, and avoiding uptake in the systemic circulation. In reality, the majority of CL patients present with open lesions which lack the epidermis and stratum corneum. Even if the epidermis is present, it is crusty and highly modified (Karram et al., 2012), (Figure 4). Depending on the physicochemical properties of the drug, especially the ability to permeate through skin layers, removal of the crust and application of a dressing could be considered in some cases. A major drawback to both “drug-like” guidelines is that they do not take into account how the pathology affects drug delivery to the target tissue in ulcerated CL lesions and/or modified epidermis including the stratum corneum on nodular lesions of CL. For the topical rule of five, these physicochemical indicators were obtained by modelling skin permeation as a series of diffusion and partition processes across the stratum corneum as the main permeation barrier. As mentioned above, they do not take into account the inflammation of the dermis and/or thickening of the epidermis, both of which are known to impact topical application (Wijnant et al., 2018), (Maleki et al., 2017). Furthermore, CL patients often present with multiple lesions at varying stages of progression (Figure

4). During the early stages, the epidermis is still present; however, little is known about the barrier capacity of the stratum corneum. In the ulcer stage, dermal skin layers are exposed and the epidermis is absent. Even though the crust is almost constantly removed in a couple days by cream applications under a dressing, during the later healing stages, there is crust formation which might complicate topical drug delivery (Karram et al., 2012).

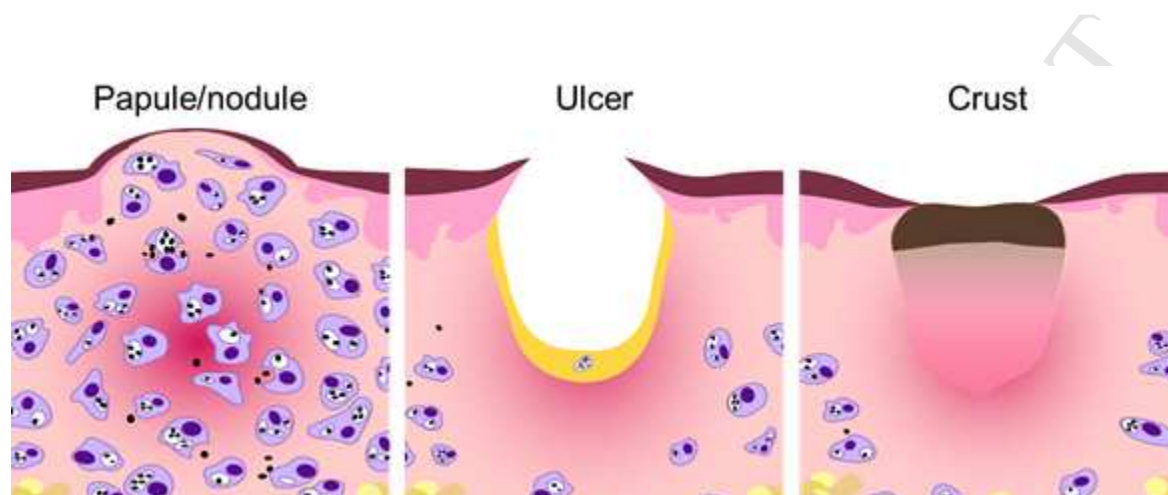


Figure 4: A schematic representation of the skin at different stages of CL. An early form of CL is shown in (A) where the skin is visibly still intact but a small nodule or papule is visible; (B) shows the more advanced stage whereby the crust was removed and an ulcer is exposed. The epidermal layers of the skin (including the stratum corneum) are absent. As part of the healing process, a crust is formed (C).

In experiences with topical paromomycin applied daily for 20 days, patients suffering from simultaneous ulcerated and nodular lesions cured of all lesions regardless of their aspect at the time of starting therapy (Ben Salah et al., 2014). These practical considerations may simplify the applicability of topical treatment with creams, despite otherwise important theoretical concerns.

Further evaluation of DMPK parameters such as compound stability (using whole hepatocytes and microsomes) and intrinsic permeability (using caco-2 and/or MDCK-MDR1 cell lines) are currently standard practice in the pharmaceutical industry. The former assays are especially important for orally administered drugs that are absorbed through the gut membrane and undergo first-pass metabolism in the liver before reaching systemic circulation. Esterases and CYP enzymes are also present in the skin and therefore might contribute to drug metabolism and excretion, even though drug metabolism in the skin is believed to occur to a far lesser extent than orally administered drugs (Baron et al., 2008). In an attempt to evaluate and identify 'red flags' for skin drug stability, candidate drugs are incubated in skin homogenate. The reduction of the parent compound fractions left at the end of the assay are then compared to the fractions of paraben drugs that are known substrates of skin esterases (Van Bocxlaer et al., 2018). Caco-2 and MDCKII-hMDR1 permeability assays are used to classify the level of permeation of test compounds crossing the gut and the blood-brain barrier (Figure 5). It also allows identification of drugs that are substrates for the P-glycoprotein efflux transporter as it potentially reduces the bioavailability of the drugs and could lead to a reduced ability to penetrate into macrophages. The evaluation of binding is important as only the unbound drug fraction is able to exert activity and thus a low bound fraction would appear

desirable. On the other hand, low drug binding to skin components is thought to reduce residence time of the drug in the skin and enhances uptake in and excretion via the lymphatic system. Some evidence suggests that minimal levels of binding to skin components is desirable and is indicative of topical drug activity. However, this work is limited to one chemical series and further studies would have to be conducted to verify this hypothesis (Van Bocxlaer et al., 2018). In addition, normal skin components may be different from lesion skin components. One pilot study has analysed the evolution of parasite loads in patients with ulcerative lesions treated with topical paromomycin applied under semi-occlusive dressings, and showed similar efficacy in superficial and deep layers of lesions (Ben Salah et al., 2014).

Despite their shortcomings, the available *in vitro* and *in vivo* CL models allow for discrimination and progression of the most promising compounds based on their skin penetration and disposition properties. As mentioned above, in the majority of CL cases in the field, the epidermis is not present and the real issue is not drug permeation of the initial skin layers but obtaining drug retention in the dermis to exert maximal antileishmanial parasite killing. In this context, drug disposition studies are important to determine which drugs penetrate and remains in the skin, whereas permeation studies can help to identify which factors thrive the process (i.e. diffusion or partition processes) which is important for formulation purposes. Drug penetration and disposition into the skin can be evaluated using a number of membranes depending on the assay hypothesis or the intended usage of the end product. Reconstructed human skin is commercially available in a 24-well plate system and requires limited set-up and equipment. This user-friendly format is therefore an attractive alternative to costly and difficult-to-obtain human skin and equally allows predictive evaluation of percutaneous permeation (Schafer-Korting et al., 2008). On the other hand, mouse skin that is less predictive of drug permeation through human skin due to physical skin differences, offers the advantage to correlate the results of permeation through a CL lesion to *in vivo* efficacy in the CL mouse model. Whilst a permeation flux similar or higher than the model skin permeants caffeine and testosterone indicates adequate permeation across the stratum corneum, also considered the main barrier to skin permeation, some retention of the drug into the skin has shown to be beneficial for topically applied drug efficacy (Van Bocxlaer et al., 2018). More importantly, the drug disposition in different skin layers can be evaluated at the end of the experiment by removing and retrieving the drug left on the skin from the epidermal layers by tape stripping and extracting the drug from the lower dermal layers to evaluate the amount retained in the skin.

To further complicate matters, a drug is rarely administered as the active pharmaceutical ingredient only. Instead, it is incorporated in a suitable carrier system composed of excipients including solvents and penetration enhancers. Some of these individual components and/or their combinations have been reported to influence the permeation of the active drug into the skin. It is therefore important to evaluate the percutaneous drug penetration ideally using uninfected and infected skin in the before mentioned Franz diffusion cell type assays (Figure 5). Whilst being a helpful tool, the reproducibility of these assays can be variable. To this respect, study design, skin type used, donor and receptor medium, and experimental set up should be carefully considered (Henning et al., 2009).

Encapsulation of drugs is another useful pharmaceutical strategy for topical CL treatment as it can offer enhanced permeation of the active drug, immunomodulatory effects, and macrophage-targeted delivery (Moreno et al., 2014), (Parra et al., 2018). Considering the complex cellular and tissue interactions of these particles, the evaluation of skin penetration requires extensive model optimization and combinations of sensitive imaging and detection methods such as Raman



spectroscopy, X-ray microscopy, and flow cytometry in addition to the Franz diffusion cell assays (Vogt et al., 2014).

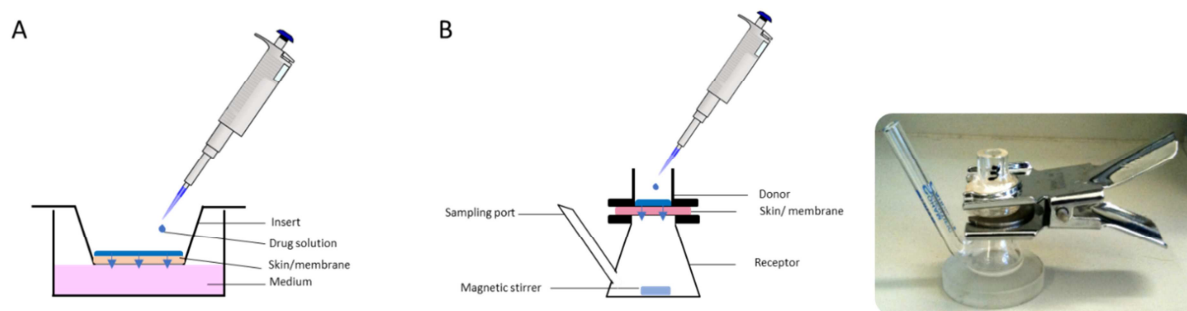


Figure 5. A schematic representation of a permeation assay using a 24-well plate system (A) or a Franz diffusion cell (b)

### Models for Immunomodulators

Immunotherapy is an alternative approach to the treatment of CL patients, particularly for those who cannot use pentavalent antimonials such as patients with nephropathy or cardiopathy. Cost and applicability have been major issues for real-life use in CL, but progress in this field may give rise to very short courses (ex. one or two injections of long-lived antibodies) at reduced costs. A recent review has detailed the immunotherapeutic approaches, including therapy with antibodies, cytokines, and vaccines (Taslimi et al., 2018). In clinical studies, immunotherapeutics have been used successfully in combinations with interferon- $\gamma$  as well as small molecules and standard anti-leishmanial compounds (Dalton and Kaye, 2010), (Convit et al., 2003), (Sundar et al., 1994), (Taslimi et al., 2018). However, efficacy has not been constant and at least 2 controlled studies have shown no effect of interferon, and several studies using imiquimod in CL have shown no or marginal superiority over reference treatments (Harms et al., 1991), (Arana et al., 1994), (Firooz et al., 2006), (Miranda-Verastegui et al., 2009). Progress in this field would require a better understanding of immune mechanisms of immunopathology and spontaneous cure in each major form of human CL. Relatively simple paradigms raised in mouse models may not yet deconvolute the full complexity of human CL.

For CL species, both small and large molecule immunomodulators have been studied *in vitro* using various macrophage models, alone and in combinations (Zahedifard and Rafati, 2018). Of the macrophage models available, bone-marrow derived macrophages have proved to be most useful as they (i) represent a more homogenous population of cells than peritoneal exudate cells or monocyte derived cell lines, (ii) show full range of responses to stimulation compared to cell lines, and (iii) significantly higher phagosomal functions such as acidification and proteolysis in compared to cell lines (Guo et al., 2015). For *in vivo* studies, both BALB/c and C57BL/6 mice have been used as experimental models. For CL, the BALB/c mice are the most widely used. Inbred mouse models have the advantage of (i) reproducibility, (ii) identification of precise mechanism of action as the immune pathways have been well-defined, (iii) faster readout, and (iv) more accessible reagents for any manipulation to show the proof of concept. At the same time, caution has to be exerted over the

interpretation of data from rodent model studies and translation to human use, as there are defined phylogenetic and immunopathology differences, in particular TLR and NLR gene repertoire differences influence their function (Ariffin and Sweet, 2013).

Recent studies illustrate the use of animal models in the development of compounds with immunomodulatory properties. Antimicrobial peptides (AMPs) can induce tissues and specific cells to regulate gene expression and secretion of cytokines and chemokines, wound healing, as well as anti or pro inflammatory effects, with activity against different species of *Leishmania* both *in vitro* as well as animal models (Abdossamadi et al., 2016). In addition, oligodeoxynucleotides (ODN's) containing unmethylated CpG motifs which mimic microbial DNA and are recognized by toll-like receptor (TLR) 9, trigger B cell activation and cytokine production as well as stimulate maturation and activation of plasmacytoid dendritic cells and production of pro-inflammatory cytokines such as IFN- $\gamma$  and IL-12, (similar to mouse model) (Verthelyi et al., 2002). These properties make them useful, amongst others, for treatment of infectious diseases (Leifer et al., 2003). Intradermal (ID) administration of CPG ODN type D/A at the site of *L. major* infections, respectively 3 days before and 3 days after and 10 days after infection with  $1 \times 10^7$  metacyclic promastigotes, reduced the severity of *L. major* caused lesions in rhesus macaque monkeys (Flynn et al., 2005). In the same study, a single treatment dose of CPG ODN administered systemically (sub cutaneous) two weeks post infection with  $2 \times 10^6$  metacyclic *L. major* parasites significantly reduced lesion size in rhesus macaque monkeys (Flynn et al., 2005). In a study conducted in 2002, co-administration of CpG ODN with non-viable *L. amazonensis* parasites provided significantly increased protection in rhesus macaque monkeys infected ID with *L. major* parasites compared to the control group (Verthelyi et al., 2002). A range of small molecules, for example, a cream of the TLR-4 agonist imiquimod (an imidazoquinoline), is active *in vivo* in *L. major* infected BALB/c mice (Buates and Matlashewski, 1999). In a novel *ex vivo* model, the presence of immunomodulators CpG and pentoxifylline modified the host immune response and affected the paracidal activity of meglumine antimoniate and miltefosine, such "underscoring the importance of pre-clinical evaluation of immunotherapeutic strategies" (Gonzalez-Fajardo et al., 2015).

## Conclusion

Since the last review of the CL drug R & D pathway (Modabber et al., 2007), no new effective and widely applicable drug or procedure has been developed for the treatment of CL (Modabber et al., 2007). Recommendations have been modified to reduce the proportion of patients receiving potentially toxic systemic drugs which are often administered without proper medical supervision in endemic areas (WHO 2010, WHO Manual for Case management East Mediterranean WHO region 2014). Local treatment has been shown to be applicable in a majority of patients and endorsed by experts (Morizot et al., 2013), (Blum et al., 2014), (Aronson, 2017). An aminoglycoside-containing cream has been developed through a collaborative effort funded by WRAIR, but its effectiveness, safety, and ease-of-use have not yet translated into wide availability. A painless local procedure is a better treatment for CL than painful physical methods like cryo- or thermo-therapy requiring tools and expertise and which proved unsustainable in real-life conditions, justifying the search for new topical options. However, any local therapy will have limitations. Some lesions, like the periorificial ones, are difficult to treat locally, even with a cream. The treatment of multi-lesional or disseminated forms such as PKDL as well as the prevention of metastatic MCL, can be better reached with a short oral course of an effective drug. Strong candidates, some of which show oral activity in mouse models, are in the pipeline and we are moving towards the pre-clinical pathway and criteria

to determine the best way to select those with the greatest chance of success in patients (Van Bocxlaer et al., 2019). Pre-clinical tools to assess safety and efficacy of antileishmanial agents have shown great progress over the last two decades. Their optimal use will accelerate the delivery of satisfactory treatment options for neglected patients with CL.

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Research was conducted under an approved animal use protocol in an AAALACi accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition.

**References:**

- ABDELADHIM, M., KAMHAWI, S. & VALENZUELA, J. G. 2014. What's behind a sand fly bite? The profound effect of sand fly saliva on host hemostasis, inflammation and immunity. *Infect Genet Evol*, 28, 691-703.
- ABDOSSAMADI, Z., SEYED, N. & RAFATI, S. 2016. Mammalian host defense peptides and their implication on combating Leishmania infection. *Cell Immunol*, 309, 23-31.
- ADAMS, E. R., GOMEZ, M. A., SCHESKE, L., RIOS, R., MARQUEZ, R., COSSIO, A., ALBERTINI, A., SCHALLIG, H. & SARAIVIA, N. G. 2014. Sensitive diagnosis of cutaneous leishmaniasis by lesion swab sampling coupled to qPCR. *Parasitology*, 141, 1891-7.
- ALVAR, J., VELEZ, I. D., BERN, C., HERRERO, M., DESJEUX, P., CANO, J., JANNIN, J., DEN BOER, M. & TEAM, W. H. O. L. C. 2012. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*, 7, e35671.
- ALVES, F., BILBE, G., BLESSON, S., GOYAL, V., MONNERAT, S., MOWBRAY, C., MUTHONI OUATTARA, G., PECOUL, B., RIJAL, S., RODE, J., SOLOMOS, A., STRUB-WOURGAFT, N., WASUNNA, M., WELLS, S., ZIJLSTRA, E. E., ARANA, B. & ALVAR, J. 2018. Recent Development of Visceral Leishmaniasis Treatments: Successes, Pitfalls, and Perspectives. *Clin Microbiol Rev*, 31.
- AMARAL, V. F., TEVA, A., PORROZZI, R., SILVA, A. J., PEREIRA, M. S., OLIVEIRA-NETO, M. P. & GRIMALDI, G., JR. 2001. Leishmania (Leishmania) major-infected rhesus macaques (Macaca mulatta) develop varying levels of resistance against homologous re-infections. *Mem Inst Oswaldo Cruz*, 96, 795-804.
- AMATO, V. S., TUON, F. F., SIQUEIRA, A. M., NICODEMO, A. C. & NETO, V. A. 2007. Treatment of mucosal leishmaniasis in Latin America: systematic review. *Am J Trop Med Hyg*, 77, 266-74.
- ARANA, B. A., NAVIN, T. R., ARANA, F. E., BERMAN, J. D. & ROSENKAIMER, F. 1994. Efficacy of a short course (10 days) of high-dose meglumine antimonate with or without interferon-gamma in treating cutaneous leishmaniasis in Guatemala. *Clin Infect Dis*, 18, 381-4.
- ARIFFIN, J. K. & SWEET, M. J. 2013. Differences in the repertoire, regulation and function of Toll-like Receptors and inflammasome-forming Nod-like Receptors between human and mouse. *Curr Opin Microbiol*, 16, 303-10.
- ARONSON, N. E. 2017. Addressing a clinical challenge: guidelines for the diagnosis and treatment of leishmaniasis. *BMC Med*, 15, 76.
- BAILEY, F., MONDRAGON-SHEM, K., HAINES, L. R., OLABI, A., ALORFI, A., RUIZ-POSTIGO, J. A., ALVAR, J., HOTEZ, P., ADAMS, E. R., VELEZ, I. D., AL-SALEM, W., EATON, J., ACOSTA-SERRANO, A. & MOLYNEUX, D. H. 2019. Cutaneous leishmaniasis and co-morbid major depressive disorder: A systematic review with burden estimates. *PLoS Negl Trop Dis*, 13, e0007092.

- BARON, J. M., WIEDERHOLT, T., HEISE, R., MERK, H. F. & BICKERS, D. R. 2008. Expression and function of cytochrome p450-dependent enzymes in human skin cells. *Curr Med Chem*, 15, 2258-64.
- BELKAID, Y., KAMHAWI, S., MODI, G., VALENZUELA, J., NOBEN-TRAUTH, N., ROWTON, E., RIBEIRO, J. & SACKS, D. L. 1998. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* Infection in the mouse ear dermis. *J Exp Med*, 188, 1941-1953.
- BELKAID, Y., MENDEZ, S., LIRA, R., KADAMBI, N., MILON, G. & SACKS, D. 2000. A natural model of *Leishmania major* infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. *J Immunol*, 165, 969-77.
- BEN SALAH, A., BEN MESSAOUD, N., GUEDRI, E., ZAATOUR, A., BEN ALAYA, N., BETTAIEB, J., GHARBI, A., BELHADJ HAMIDA, N., BOUKTHIR, A., CHLIF, S., ABDELHAMID, K., EL AHMADI, Z., LOUZIR, H., MOKNI, M., MORIZOT, G., BUFFET, P., SMITH, P. L., KOPYDLOWSKI, K. M., KREISHMAN-DEITRICK, M., SMITH, K. S., NIELSEN, C. J., ULLMAN, D. R., NORWOOD, J. A., THORNE, G. D., MCCARTHY, W. F., ADAMS, R. C., RICE, R. M., TANG, D., BERMAN, J., RANSOM, J., MAGILL, A. J. & GROGL, M. 2013. Topical paromomycin with or without gentamicin for cutaneous leishmaniasis. *N Engl J Med*, 368, 524-32.
- BEN SALAH, A., ZAATOUR, A., BEN MESSAOUD, N., KIDAR, A., SMITH, P. L., KOPYDLOWSKI, K. M., KREISHMAN-DEITRICK, M., NIELSEN, C. J., NOVITT-MORENO, A., RANSOM, J. H., MORIZOT, G., GROGL, M. & BUFFET, P. A. 2014. Parasite load decrease during application of a safe and easily applied antileishmanial aminoglycoside cream. *PLoS Negl Trop Dis*, 8, e2749.
- BENNIS, I., DE BROUWERE, V., BELRHITI, Z., SAHIBI, H. & BOELAERT, M. 2018. Psychosocial burden of localised cutaneous Leishmaniasis: a scoping review. *BMC Public Health*, 18, 358.
- BLUM, J., BUFFET, P., VISSER, L., HARMS, G., BAILEY, M. S., CAUMES, E., CLERINX, J., VAN THIEL, P. P., MORIZOT, G., HATZ, C., DORLO, T. P. & LOCKWOOD, D. N. 2014. LeishMan recommendations for treatment of cutaneous and mucosal leishmaniasis in travelers, 2014. *J Travel Med*, 21, 116-29.
- BOGGILD, A. K., VALENCIA, B. M., VELAND, N., PILAR RAMOS, A., CALDERON, F., AREVALO, J., LOW, D. E. & LLANOS-CUENTAS, A. 2011. Non-invasive cytology brush PCR diagnostic testing in mucosal leishmaniasis: superior performance to conventional biopsy with histopathology. *PLoS One*, 6, e26395.
- BOLHASSANI, A., TAHERI, T., TASLIMI, Y., ZAMANILUI, S., ZAHEDIFARD, F., SEYED, N., TORKASHVAND, F., VAZIRI, B. & RAFATI, S. 2011. Fluorescent *Leishmania* species: development of stable GFP expression and its application for in vitro and in vivo studies. *Exp Parasitol*, 127, 637-45.
- BUATES, S. & MATLASHEWSKI, G. 1999. Treatment of experimental leishmaniasis with the immunomodulators imiquimod and S-28463: efficacy and mode of action. *J Infect Dis*, 179, 1485-94.
- BURZA, S., CROFT, S. L. & BOELAERT, M. 2018. Leishmaniasis. *Lancet*, 392, 951-970.
- BUSSOTTI, G., GOUZELOU, E., CORTES BOITE, M., KHERACHI, I., HARRAT, Z., EDDAIKRA, N., MOTTRAM, J. C., ANTONIOU, M., CHRISTODOULOU, V., BALI, A., GUERFALI, F. Z., LAQUINI, D., MUKHTAR, M., DUMETZ, F., DUJARDIN, J. C., SMIRLIS, D., LECHAT, P.,

- PESCHER, P., EL HAMOUCHI, A., LEMRANI, M., CHICHARRO, C., LLANES-ACEVEDO, I. P., BOTANA, L., CRUZ, I., MORENO, J., JEDDI, F., AOUN, K., BOURATBINE, A., CUPOLILLO, E. & SPATH, G. F. 2018. Leishmania Genome Dynamics during Environmental Adaptation Reveal Strain-Specific Differences in Gene Copy Number Variation, Karyotype Instability, and Telomeric Amplification. *MBio*, 9.
- CALVO-ALVAREZ, E., GUERRERO, N. A., ALVAREZ-VELILLA, R., PRADA, C. F., REQUENA, J. M., PUNZON, C., LLAMAS, M. A., AREVALO, F. J., RIVAS, L., FRESNO, M., PEREZ-PERTEJO, Y., BALANA-FOUCE, R. & REGUERA, R. M. 2012. Appraisal of a Leishmania major strain stably expressing mCherry fluorescent protein for both in vitro and in vivo studies of potential drugs and vaccine against cutaneous leishmaniasis. *PLoS Negl Trop Dis*, 6, e1927.
- CALVO-ALVAREZ, E., STAMATAKIS, K., PUNZON, C., ALVAREZ-VELILLA, R., TEJERIA, A., ESCUDERO-MARTINEZ, J. M., PEREZ-PERTEJO, Y., FRESNO, M., BALANA-FOUCE, R. & REGUERA, R. M. 2015. Infrared fluorescent imaging as a potent tool for in vitro, ex vivo and in vivo models of visceral leishmaniasis. *PLoS Negl Trop Dis*, 9, e0003666.
- CARIDHA, D., PARRIOT, S., HUDSON, T. H., LANG, T., NGUNDAM, F., LEED, S., SENA, J., HARRIS, M., O'NEIL, M., SCIOTTI, R., READ, L., LECOEUR, H., HICKMAN, M. & GROGL, M. 2017. Use of Optical Imaging Technology in the Validation of a New, Rapid, Cost-Effective Drug Screen as Part of a Tiered In Vivo Screening Paradigm for Development of Drugs To Treat Cutaneous Leishmaniasis. *Antimicrob Agents Chemother*, 61.
- CAWLFIELD, A., VESELY, B., NGUNDAM, F., BUTLER, K., NUGENT, D., ROWLAND, T. & CARIDHA, D. 2018. Use of In Vivo Imaging System Technology in Leishmania major BALB/c Mouse Ear Infection Studies. *J Med Entomol*, 55, 429-435.
- COELHO, A. C., OLIVEIRA, J. C., ESPADA, C. R., REIMAO, J. Q., TRINCONI, C. T. & ULIANA, S. R. 2016. A Luciferase-Expressing Leishmania braziliensis Line That Leads to Sustained Skin Lesions in BALB/c Mice and Allows Monitoring of Miltefosine Treatment Outcome. *PLoS Negl Trop Dis*, 10, e0004660.
- CONVIT, J., PINARDI, M. E. & RONDON, A. J. 1972. Diffuse cutaneous leishmaniasis: a disease due to an immunological defect of the host. *Trans R Soc Trop Med Hyg*, 66, 603-10.
- CONVIT, J., ULRICH, M., ZERPA, O., BORGES, R., ARANZAZU, N., VALERA, M., VILLARROEL, H., ZAPATA, Z. & TOMEDES, I. 2003. Immunotherapy of american cutaneous leishmaniasis in Venezuela during the period 1990-99. *Trans R Soc Trop Med Hyg*, 97, 469-72.
- COPELAND, N. K. & ARONSON, N. E. 2015. Leishmaniasis: treatment updates and clinical practice guidelines review. *Curr Opin Infect Dis*, 28, 426-37.
- COSTA, R. S., CARVALHO, L. P., CAMPOS, T. M., MAGALHAES, A. S., PASSOS, S. T., SCHRIEFER, A., SILVA, J. A., LAGO, E., PAIXAO, C. S., MACHADO, P., SCOTT, P. & CARVALHO, E. M. 2018. Early Cutaneous Leishmaniasis Patients Infected With Leishmania braziliensis Express Increased Inflammatory Responses After Antimony Therapy. *J Infect Dis*, 217, 840-850.
- COTA, G. F., DE SOUSA, M. R., FERREQUETTI, T. O., SALEME, P. S., ALVARISA, T. K. & RABELLO, A. 2016. The Cure Rate after Placebo or No Therapy in American Cutaneous Leishmaniasis: A Systematic Review and Meta-Analysis. *PLoS One*, 11, e0149697.
- CROFT, S. 2017. Design of treatments for cutaneous and visceral leishmaniasis. *Nihon Yakurigaku Zasshi*, 149, 220-224.



- CROFT, S. L., SEIFERT, K. & YARDLEY, V. 2006. Current scenario of drug development for leishmaniasis. *Indian J Med Res*, 123, 399-410.
- DALTON, J. E. & KAYE, P. M. 2010. Immunomodulators: use in combined therapy against leishmaniasis. *Expert Rev Anti Infect Ther*, 8, 739-42.
- DAVID, C. V. & CRAFT, N. 2009. Cutaneous and mucocutaneous leishmaniasis. *Dermatol Ther*, 22, 491-502.
- DE RYCKER, M., HALLYBURTON, I., THOMAS, J., CAMPBELL, L., WYLLIE, S., JOSHI, D., CAMERON, S., GILBERT, I. H., WYATT, P. G., FREARSON, J. A., FAIRLAMB, A. H. & GRAY, D. W. 2013. Comparison of a high-throughput high-content intracellular *Leishmania donovani* assay with an axenic amastigote assay. *Antimicrob Agents Chemother*, 57, 2913-22.
- DOSTALOVA, A. & VOLF, P. 2012. *Leishmania* development in sand flies: parasite-vector interactions overview. *Parasit Vectors*, 5, 276.
- DUBE, A., GUPTA, R. & SINGH, N. 2009. Reporter genes facilitating discovery of drugs targeting protozoan parasites. *Trends Parasitol*, 25, 432-9.
- FILONOV, G. S., PIATKEVICH, K. D., TING, L. M., ZHANG, J., KIM, K. & VERKHUSHA, V. V. 2011. Bright and stable near-infrared fluorescent protein for in vivo imaging. *Nat Biotechnol*, 29, 757-61.
- FIROOZ, A., KHAMESIPOUR, A., GHOORCHI, M. H., NASSIRI-KASHANI, M., ESKANDARI, S. E., KHATAMI, A., HOOSHMAND, B., GOROUHI, F., RASHIGHI-FIROOZABADI, M. & DOWLATI, Y. 2006. Imiquimod in combination with meglumine antimoniate for cutaneous leishmaniasis: a randomized assessor-blind controlled trial. *Arch Dermatol*, 142, 1575-9.
- FLYNN, B., WANG, V., SACKS, D. L., SEDER, R. A. & VERTHELYI, D. 2005. Prevention and treatment of cutaneous leishmaniasis in primates by using synthetic type D/A oligodeoxynucleotides expressing CpG motifs. *Infect Immun*, 73, 4948-54.
- FREIDAG, B. L., MENDEZ, S., CHEEVER, A. W., KENNEY, R. T., FLYNN, B., SACKS, D. L. & SEDER, R. A. 2003. Immunological and pathological evaluation of rhesus macaques infected with *Leishmania major*. *Exp Parasitol*, 103, 160-8.
- GAMBOA, D., TORRES, K., DE DONCKER, S., ZIMIC, M., AREVALO, J. & DUJARDIN, J. C. 2008. Evaluation of an in vitro and in vivo model for experimental infection with *Leishmania (Viannia) braziliensis* and *L. (V.) peruviana*. *Parasitology*, 135, 319-26.
- GITHURE, J. I., REID, G. D., BINHAZIM, A. A., ANJILI, C. O., SHATRY, A. M. & HENDRICKS, L. D. 1987. *Leishmania major*: the suitability of East African nonhuman primates as animal models for cutaneous leishmaniasis. *Exp Parasitol*, 64, 438-47.
- GOMES-SILVA, A., VALVERDE, J. G., RIBEIRO-ROMAO, R. P., PLACIDO-PEREIRA, R. M. & DA-CRUZ, A. M. 2013. Golden hamster (*Mesocricetus auratus*) as an experimental model for *Leishmania (Viannia) braziliensis* infection. *Parasitology*, 140, 771-9.
- GONZALEZ-FAJARDO, L., FERNANDEZ, O. L., MCMAHON-PRATT, D. & SARAIVA, N. G. 2015. Ex vivo host and parasite response to antileishmanial drugs and immunomodulators. *PLoS Negl Trop Dis*, 9, e0003820.
- GONZALEZ, U., PINART, M., RENGIFO-PARDO, M., MACAYA, A., ALVAR, J. & TWEED, J. A. 2009. Interventions for American cutaneous and mucocutaneous leishmaniasis. *Cochrane Database Syst Rev*, CD004834.
- GONZALEZ, U., PINART, M., REVEIZ, L. & ALVAR, J. 2008. Interventions for Old World cutaneous leishmaniasis. *Cochrane Database Syst Rev*, CD005067.

- GONZALEZ, U., PINART, M., REVEIZ, L., RENGIFO-PARDO, M., TWEED, J., MACAYA, A. & ALVAR, J. 2010. Designing and reporting clinical trials on treatments for cutaneous leishmaniasis. *Clin Infect Dis*, 51, 409-19.
- GROGL, M., HICKMAN, M., ELLIS, W., HUDSON, T., LAZO, J. S., SHARLOW, E. R., JOHNSON, J., BERMAN, J. & SCIOTTI, R. J. 2013. Drug discovery algorithm for cutaneous leishmaniasis. *Am J Trop Med Hyg*, 88, 216-21.
- GROGL, M., SCHUSTER, B. G., ELLIS, W. Y. & BERMAN, J. D. 1999. Successful topical treatment of murine cutaneous leishmaniasis with a combination of paromomycin (Aminosidine) and gentamicin. *J Parasitol*, 85, 354-9.
- GUERY, R., HENRY, B., MARTIN-BLONDEL, G., ROUZAUD, C., CORDOLIANI, F., HARMS, G., GANGNEUX, J. P., FOULET, F., BOURRAT, E., BACCARD, M., MORIZOT, G., CONSIGNY, P. H., BERRY, A., BLUM, J., LORTHOLARY, O., BUFFET, P., FRENCH CUTANEOUS LEISHMANIASIS STUDY, G. & THE LEISHMAN, N. 2017. Liposomal amphotericin B in travelers with cutaneous and muco-cutaneous leishmaniasis: Not a panacea. *PLoS Negl Trop Dis*, 11, e0006094.
- GUO, M., HARTLOVA, A., DILL, B. D., PRESCOTT, A. R., GIERLINSKI, M. & TROST, M. 2015. High-resolution quantitative proteome analysis reveals substantial differences between phagosomes of RAW 264.7 and bone marrow derived macrophages. *Proteomics*, 15, 3169-74.
- GUPTA, S. & NISHI 2011. Visceral leishmaniasis: experimental models for drug discovery. *Indian J Med Res*, 133, 27-39.
- HARMS, G., CHEHADE, A. K., DOUBA, M., ROEPKE, M., MOUAKEH, A., ROSENKAIMER, F. & BIENZLE, U. 1991. A randomized trial comparing a pentavalent antimonial drug and recombinant interferon-gamma in the local treatment of cutaneous leishmaniasis. *Trans R Soc Trop Med Hyg*, 85, 214-6.
- HENNING, A., SCHAEFER, U. F. & NEUMANN, D. 2009. Potential pitfalls in skin permeation experiments: influence of experimental factors and subsequent data evaluation. *Eur J Pharm Biopharm*, 72, 324-31.
- HORVATH, P., AULNER, N., BICKLE, M., DAVIES, A. M., NERY, E. D., EBNER, D., MONTOYA, M. C., OSTLING, P., PIETIAINEN, V., PRICE, L. S., SHORTE, S. L., TURCATTI, G., VON SCHANTZ, C. & CARRAGHER, N. O. 2016. Screening out irrelevant cell-based models of disease. *Nat Rev Drug Discov*, 15, 751-769.
- KARRAM, S., LOYA, A., HAMAM, H., HABIB, R. H. & KHALIFEH, I. 2012. Transepidermal elimination in cutaneous leishmaniasis: a multiregional study. *J Cutan Pathol*, 39, 406-12.
- KIMBLIN, N., PETERS, N., DEBRABANT, A., SECUNDINO, N., EGEN, J., LAWYER, P., FAY, M. P., KAMHAWI, S. & SACKS, D. 2008. Quantification of the infectious dose of *Leishmania major* transmitted to the skin by single sand flies. *Proc Natl Acad Sci U S A*, 105, 10125-30.
- LAMOTTE, S., SPATH, G. F., RACHIDI, N. & PRINA, E. 2017. The enemy within: Targeting host-parasite interaction for antileishmanial drug discovery. *PLoS Negl Trop Dis*, 11, e0005480.
- LECOEUR, H., BUFFET, P., MORIZOT, G., GOYARD, S., GUIGON, G., MILON, G. & LANG, T. 2007. Optimization of topical therapy for *Leishmania major* localized cutaneous leishmaniasis using a reliable C57BL/6 Model. *PLoS Negl Trop Dis*, 1, e34.
- LECOEUR, H., BUFFET, P. A., MILON, G. & LANG, T. 2010. Early curative applications of the aminoglycoside WR279396 on an experimental *Leishmania major*-loaded cutaneous



- site do not impair the acquisition of immunity. *Antimicrob Agents Chemother*, 54, 984-90.
- LEE, S. A. & HASBUN, R. 2003. Therapy of cutaneous leishmaniasis. *Int J Infect Dis*, 7, 86-93.
- LEIFER, C. A., VERTHELYI, D. & KLINMAN, D. M. 2003. Heterogeneity in the human response to immunostimulatory CpG oligodeoxynucleotides. *J Immunother*, 26, 313-9.
- LIPINSKI, C. A., LOMBARDO, F., DOMINY, B. W. & FEENEY, P. J. 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev*, 46, 3-26.
- LOEUILLET, C., BANULS, A. L. & HIDE, M. 2016. Study of Leishmania pathogenesis in mice: experimental considerations. *Parasit Vectors*, 9, 144.
- LORIA-CERVERA, E. N. & ANDRADE-NARVAEZ, F. J. 2014. Animal models for the study of leishmaniasis immunology. *Rev Inst Med Trop Sao Paulo*, 56, 1-11.
- MACHADO, G. U., PRATES, F. V. & MACHADO, P. R. L. 2019. Disseminated leishmaniasis: clinical, pathogenic, and therapeutic aspects. *An Bras Dermatol*, 94, 9-16.
- MALEKI, M., YOUSEFI, M., BAZZAZ, S. M. M., TABASSI, S. A. S., RAKHSHANDEH, H., HAMED, S. S. & HAGHIGHI, G. 2017. An overview of skin lesions adapted to Cutaneous Leishmaniasis in Persian Medicine. *Electron Physician*, 9, 5854-5862.
- MCKERROW, J. H. & LIPINSKI, C. A. 2017. The rule of five should not impede anti-parasitic drug development. *Int J Parasitol Drugs Drug Resist*, 7, 248-249.
- MEARS, E. R., MODABBER, F., DON, R. & JOHNSON, G. E. 2015. A Review: The Current In Vivo Models for the Discovery and Utility of New Anti-leishmanial Drugs Targeting Cutaneous Leishmaniasis. *PLoS Negl Trop Dis*, 9, e0003889.
- MEHTA, S. R., HUANG, R., YANG, M., ZHANG, X. Q., KOLLI, B., CHANG, K. P., HOFFMAN, R. M., GOTO, Y., BADARO, R. & SCHOOLEY, R. T. 2008. Real-time in vivo green fluorescent protein imaging of a murine leishmaniasis model as a new tool for Leishmania vaccine and drug discovery. *Clin Vaccine Immunol*, 15, 1764-70.
- MILLINGTON, O. R., MYBURGH, E., MOTTRAM, J. C. & ALEXANDER, J. 2010. Imaging of the host/parasite interplay in cutaneous leishmaniasis. *Exp Parasitol*, 126, 310-7.
- MILON, G., DEL GIUDICE, G. & LOUIS, J. A. 1995. Immunobiology of experimental cutaneous leishmaniasis. *Parasitol Today*, 11, 244-7.
- MIRANDA-VERASTEGUI, C., TULLIANO, G., GYORKOS, T. W., CALDERON, W., RAHME, E., WARD, B., CRUZ, M., LLANOS-CUENTAS, A. & MATLASHEWSKI, G. 2009. First-line therapy for human cutaneous leishmaniasis in Peru using the TLR7 agonist imiquimod in combination with pentavalent antimony. *PLoS Negl Trop Dis*, 3, e491.
- MODABBER, F., BUFFET, P. A., TORREELE, E., MILON, G. & CROFT, S. L. 2007. Consultative meeting to develop a strategy for treatment of cutaneous leishmaniasis. Institute Pasteur, Paris. 13-15 June, 2006. *Kinetoplastid Biol Dis*, 6, 3.
- MORENO, E., SCHWARTZ, J., FERNANDEZ, C., SANMARTIN, C., NGUEWA, P., IRACHE, J. M. & ESPUELAS, S. 2014. Nanoparticles as multifunctional devices for the topical treatment of cutaneous leishmaniasis. *Expert Opin Drug Deliv*, 11, 579-97.
- MORIZOT, G., KENDJO, E., MOURI, O., THELLIER, M., PERIGNON, A., FOULET, F., CORDOLIANI, F., BOURRAT, E., LAFFITTE, E., ALCARAZ, I., BODAK, N., RAVEL, C., VRAY, M., GROGL, M., MAZIER, D., CAUMES, E., LACHAUD, L., BUFFET, P. A. & CUTANEOUS LEISHMANIASIS FRENCH STUDY, G. 2013. Travelers with cutaneous leishmaniasis cured without systemic therapy. *Clin Infect Dis*, 57, 370-80.
- NAIK, A., KALIA, Y. N. & GUY, R. H. 2000. Transdermal drug delivery: overcoming the skin's barrier function. *Pharm Sci Technol Today*, 3, 318-326.

- OLLIARO, P., GROGL, M., BONI, M., CARVALHO, E. M., CHEBLI, H., CISSE, M., DIRO, E., FERNANDES COTA, G., ERBER, A. C., GADISA, E., HANDJANI, F., KHAMESIPOUR, A., LLANOS-CUENTAS, A., LOPEZ CARVAJAL, L., GROUT, L., LMIMOUNI, B. E., MOKNI, M., NAHZAT, M. S., BEN SALAH, A., OZBEL, Y., PASCALE, J. M., RIZZO MOLINA, N., RODE, J., ROMERO, G., RUIZ-POSTIGO, J. A., GORE SARAVIA, N., SOTO, J., UZUN, S., MASHAYEKHI, V., VELEZ, I. D., VOGT, F., ZERPA, O. & ARANA, B. 2018. Harmonized clinical trial methodologies for localized cutaneous leishmaniasis and potential for extensive network with capacities for clinical evaluation. *PLoS Negl Trop Dis*, 12, e0006141.
- OLLIARO, P., VAILLANT, M., ARANA, B., GROGL, M., MODABBER, F., MAGILL, A., LAPUJADE, O., BUFFET, P. & ALVAR, J. 2013. Methodology of clinical trials aimed at assessing interventions for cutaneous leishmaniasis. *PLoS Negl Trop Dis*, 7, e2130.
- PARRA, F. L., CAIMI, A. T., ALTUBE, M. J., CARGNELUTTI, D. E., VERMEULEN, M. E., DE FARIAS, M. A., PORTUGAL, R. V., MORILLA, M. J. & ROMERO, E. L. 2018. Make It Simple: (SR-A1+TLR7) Macrophage Targeted NANOarchaeosomes. *Front Bioeng Biotechnol*, 6, 163.
- PROBST, R. J., WELLDE, B. T., LAWYER, P. G., STITELER, J. S. & ROWTON, E. D. 2001. Rhesus monkey model for *Leishmania major* transmitted by *Phlebotomus papatasi* sandfly bites. *Med Vet Entomol*, 15, 12-21.
- PULIDO, S. A., MUNOZ, D. L., RESTREPO, A. M., MESA, C. V., ALZATE, J. F., VELEZ, I. D. & ROBLEDO, S. M. 2012. Improvement of the green fluorescent protein reporter system in *Leishmania* spp. for the in vitro and in vivo screening of antileishmanial drugs. *Acta Trop*, 122, 36-45.
- RAHAVENDRAN, S. V., VEKICH, S., SKOR, H., BATUGO, M., NGUYEN, L., SHETTY, B. & SHEN, Z. 2012. Discovery pharmacokinetic studies in mice using serial microsampling, dried blood spots and microbore LC-MS/MS. *Bioanalysis*, 4, 1077-95.
- REIMAO, J. Q., TRINCONI, C. T., YOKOYAMA-YASUNAKA, J. K., MIGUEL, D. C., KALIL, S. P. & ULIANA, S. R. 2013. Parasite burden in *Leishmania* (*Leishmania*) *amazonensis*-infected mice: validation of luciferase as a quantitative tool. *J Microbiol Methods*, 93, 95-101.
- RIBEIRO-ROMAO, R. P., MOREIRA, O. C., OSORIO, E. Y., CYSNE-FINKELSTEIN, L., GOMES-SILVA, A., VALVERDE, J. G., PIRMEZ, C., DA-CRUZ, A. M. & PINTO, E. F. 2014. Comparative evaluation of lesion development, tissue damage, and cytokine expression in golden hamsters (*Mesocricetus auratus*) infected by inocula with different *Leishmania* (*Viannia*) *braziliensis* concentrations. *Infect Immun*, 82, 5203-13.
- ROBLEDO, S. M., CARRILLO, L. M., DAZA, A., RESTREPO, A. M., MUNOZ, D. L., TOBON, J., MURILLO, J. D., LOPEZ, A., RIOS, C., MESA, C. V., UPEGUI, Y. A., VALENCIA-TOBON, A., MONDRAGON-SHEM, K., RODRIGUEZ, B. & VELEZ, I. D. 2012. Cutaneous leishmaniasis in the dorsal skin of hamsters: a useful model for the screening of antileishmanial drugs. *J Vis Exp*.
- ROCHA, M. N., CORREA, C. M., MELO, M. N., BEVERLEY, S. M., MARTINS-FILHO, O. A., MADUREIRA, A. P. & SOARES, R. P. 2013. An alternative in vitro drug screening test using *Leishmania amazonensis* transfected with red fluorescent protein. *Diagn Microbiol Infect Dis*, 75, 282-91.
- ROY, G., DUMAS, C., SERENO, D., WU, Y., SINGH, A. K., TREMBLAY, M. J., OUELLETTE, M., OLIVIER, M. & PAPADOPOULOU, B. 2000. Episomal and stable expression of the

- luciferase reporter gene for quantifying *Leishmania* spp. infections in macrophages and in animal models. *Mol Biochem Parasitol*, 110, 195-206.
- SCHAFFER-KORTING, M., BOCK, U., DIEMBECK, W., DUSING, H. J., GAMER, A., HALTNER-UKOMADU, E., HOFFMANN, C., KACA, M., KAMP, H., KERSEN, S., KIETZMANN, M., KORTING, H. C., KRACHTER, H. U., LEHR, C. M., LIEBSCH, M., MEHLING, A., MULLER-GOYMANN, C., NETZLAFF, F., NIEDORF, F., RUBBELKE, M. K., SCHAFFER, U., SCHMIDT, E., SCHREIBER, S., SPIELMANN, H., VUIA, A. & WEIMER, M. 2008. The use of reconstructed human epidermis for skin absorption testing: Results of the validation study. *Altern Lab Anim*, 36, 161-87.
- SCHUSTER, S., HARTLEY, M. A., TACCHINI-COTTIER, F. & RNET, C. 2014. A scoring method to standardize lesion monitoring following intra-dermal infection of *Leishmania* parasites in the murine ear. *Front Cell Infect Microbiol*, 4, 67.
- SCOTT, P. & NOVAIS, F. O. 2016. Cutaneous leishmaniasis: immune responses in protection and pathogenesis. *Nat Rev Immunol*, 16, 581-92.
- SEIFERT, K., ESCOBAR, P. & CROFT, S. L. 2010. In vitro activity of anti-leishmanial drugs against *Leishmania donovani* is host cell dependent. *J Antimicrob Chemother*, 65, 508-11.
- SIQUEIRA-NETO, J. L., MOON, S., JANG, J., YANG, G., LEE, C., MOON, H. K., CHATELAIN, E., GENOVESIO, A., CECHETTO, J. & FREITAS-JUNIOR, L. H. 2012. An image-based high-content screening assay for compounds targeting intracellular *Leishmania donovani* amastigotes in human macrophages. *PLoS Negl Trop Dis*, 6, e1671.
- SOLOMON, M., PAVLOTZKY, F., BARZILAI, A. & SCHWARTZ, E. 2013. Liposomal amphotericin B in comparison to sodium stibogluconate for *Leishmania braziliensis* cutaneous leishmaniasis in travelers. *J Am Acad Dermatol*, 68, 284-9.
- SUAREZ, M., VALENCIA, B. M., JARA, M., ALBA, M., BOGGILD, A. K., DUJARDIN, J. C., LLANOS-CUENTAS, A., AREVALO, J. & ADAUI, V. 2015. Quantification of *Leishmania* (Viannia) Kinetoplast DNA in Ulcers of Cutaneous Leishmaniasis Reveals Inter-site and Inter-sampling Variability in Parasite Load. *PLoS Negl Trop Dis*, 9, e0003936.
- SUNDAR, S., ROSENKAIMER, F. & MURRAY, H. W. 1994. Successful treatment of refractory visceral leishmaniasis in India using antimony plus interferon-gamma. *J Infect Dis*, 170, 659-62.
- TASLIMI, Y., ZAHEDIFARD, F. & RAFATI, S. 2018. Leishmaniasis and various immunotherapeutic approaches. *Parasitology*, 145, 497-507.
- TEGAZZINI, D., DIAZ, R., AGUILAR, F., PENA, I., PRESA, J. L., YARDLEY, V., MARTIN, J. J., COTERON, J. M., CROFT, S. L. & CANTIZANI, J. 2016. A Replicative In Vitro Assay for Drug Discovery against *Leishmania donovani*. *Antimicrob Agents Chemother*, 60, 3524-32.
- UNGER, A., O'NEAL, S., MACHADO, P. R., GUIMARAES, L. H., MORGAN, D. J., SCHRIEFER, A., BACELLAR, O., GLESBY, M. J. & CARVALHO, E. M. 2009. Association of treatment of American cutaneous leishmaniasis prior to ulcer development with high rate of failure in northeastern Brazil. *Am J Trop Med Hyg*, 80, 574-9.
- VAN BOCXLAER, K., GAUKEL, E., HAUSER, D., PARK, S. H., SCHOCK, S., YARDLEY, V., RANDOLPH, R., PLATTNER, J. J., MERCHANT, T., CROFT, S. L., JACOBS, R. T. & WRING, S. A. 2018. Topical Treatment for Cutaneous Leishmaniasis: Dermato-Pharmacokinetic Lead Optimization of Benzoxaboroles. *Antimicrob Agents Chemother*, 62.

- VAN DEN KERKHOFF, M., MABILLE, D., CHATELAIN, E., MOWBRAY, C. E., BRAILLARD, S., HENDRICKX, S., MAES, L. & CALJON, G. 2018. In vitro and in vivo pharmacodynamics of three novel antileishmanial lead series. *Int J Parasitol Drugs Drug Resist*, 8, 81-86.
- VAN THIEL, P. P., LEENSTRA, T., KAGER, P. A., DE VRIES, H. J., VAN VUGT, M., VAN DER MEIDE, W. F., BART, A., ZEEGELAAR, J. E., VAN DER SLUIS, A., SCHALLIG, H. D., VAN GOOL, T., FABER, W. R. & DE VRIES, P. J. 2010. Miltefosine treatment of *Leishmania major* infection: an observational study involving Dutch military personnel returning from northern Afghanistan. *Clin Infect Dis*, 50, 80-3.
- VELEZ, I., LOPEZ, L., SANCHEZ, X., MESTRA, L., ROJAS, C. & RODRIGUEZ, E. 2010. Efficacy of miltefosine for the treatment of American cutaneous leishmaniasis. *Am J Trop Med Hyg*, 83, 351-6.
- VERMEERSCH, M., DA LUZ, R. I., TOTE, K., TIMMERMANS, J. P., COS, P. & MAES, L. 2009. In vitro susceptibilities of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. *Antimicrob Agents Chemother*, 53, 3855-9.
- VERTHELYI, D., KENNEY, R. T., SEDER, R. A., GAM, A. A., FRIEDAG, B. & KLINMAN, D. M. 2002. CpG oligodeoxynucleotides as vaccine adjuvants in primates. *J Immunol*, 168, 1659-63.
- VOGT, A., RANCAN, F., AHLBERG, S., NAZEMI, B., CHOE, C. S., DARVIN, M. E., HADAM, S., BLUME-PEYTAVI, U., LOZA, K., DIENDORF, J., EPPLE, M., GRAF, C., RUHL, E., MEINKE, M. C. & LADEMANN, J. 2014. Interaction of dermatologically relevant nanoparticles with skin cells and skin. *Beilstein J Nanotechnol*, 5, 2363-73.
- WEGE, A. K., FLORIAN, C., ERNST, W., ZIMARA, N., SCHLEICHER, U., HANSES, F., SCHMID, M. & RITTER, U. 2012. *Leishmania major* infection in humanized mice induces systemic infection and provokes a nonprotective human immune response. *PLoS Negl Trop Dis*, 6, e1741.
- WIJNANT, G. J., VAN BOCXLAER, K., FORTES FRANCISCO, A., YARDLEY, V., HARRIS, A., ALAVIJEH, M., MURDAN, S. & CROFT, S. L. 2018. Local Skin Inflammation in Cutaneous Leishmaniasis as a Source of Variable Pharmacokinetics and Therapeutic Efficacy of Liposomal Amphotericin B. *Antimicrob Agents Chemother*, 62.
- ZAHEDIFARD, F. & RAFATI, S. 2018. Prospects for antimicrobial peptide-based immunotherapy approaches in *Leishmania* control. *Expert Rev Anti Infect Ther*, 16, 461-469.
- ZULFIQAR, B., SHELPER, T. B. & AVERY, V. M. 2017. Leishmaniasis drug discovery: recent progress and challenges in assay development. *Drug Discov Today*, 22, 1516-1531.

**Conflicts of Interest**

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Research was conducted under an approved animal use protocol in an AAALACi accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition.