Leishmania and other intracellular pathogens: selectivity, drug distribution and PK PD

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Abstract

New drugs and treatments for diseases caused by intracellular pathogens, such as leishmaniasis and the *Leishmania* species, have proved to be some of the most difficult to discover and develop. The focus of discovery research has been on the identification of potent and selective compounds that inhibit target enzymes ~~(~~or other essential molecules~~)~~ or are active against the causative pathogen in phenotypic *in vitro* assays. Although these discovery paradigms remain an essential part of the early stages of the drug R & D pathway, over the past two decades additional emphasis has been given to the challenges needed to ensure that the potential anti-infective drugs distribute to infected tissues, reach the target pathogen within the host cell and exert the appropriate pharmacodynamic effect at these sites. This review will focus on how these challenges are being met in relation to *Leishmania* and the leishmaniasis with lessons learned from drug R & D for other intracellular pathogens.

Key words: *Leishmania*, pharmacodynamics, pharmacokinetics, drug distribution

Introduction

In 2017 a small collection of drugs and treatments are used and recommended for visceral leishmaniasis (VL) (WHO, 2010; Aronson et al., 2016) and even fewer that have proven effective in the treatment of cutaneous leishmaniasis (CL) (Gonzalez et al. 2008, 2009; Aronson et al. 2016). Of these, and still widely in use, are the pentavalent antimonials (Pentostam has been in use since the 1940s; Goodwin, 1995), amphotericin B which was first used for leishmaniasis in 1960 (Sampaio et al.,196o) and as a liposomal formulation in 1991 (Davidson et al 1991), paromomycin first used for leishmaniasis in 1963 (Kellina, 1966), and miltefosine identified as anti-leishmanial in 1984 (Croft et al. 1987) and registered for use for VL treatment in 2002 (Sundar et al. 2012). The limitations of these drugs and

treatments have been reviewed elsewhere (Croft, Olliaro, 2011; Aronson et al. 2016). It is worth noting that these drugs providing the standard treatments for VL and CL are mainly re-purposed. To help improve the drug R & D process it is important to ask why it is taking so long to identify purpose-designed anti-leishmanials.

Approaches to the design, discovery and development of anti-infectives have advanced considerably over the past two decades and several unique molecular and biochemical targets of parasites/microbes are the subject of other articles in this volume [Editor to include REFS]. For leishmaniasis advances in molecular biology and structural biology have similarly led the elaboration of validated targets and inhibitors (Gilbert, 2013; Horn and Duraisingh, 2014), whilst high-throughput (HTS) and high content (HCS) have led to the identification of novel chemical series (Siquiera-Neto et al. 2012; Pena et al. 2015), as well as the identification of novel targets (Khare et al. 2016). A pragmatic use of screening and extension of chemical series with known anti-kinetoplastid activity has produced lead compounds, and novel chemical entities (NCEs), from oxaboroles, nitroimidazoles, aminopyrazoles; which are all promising candidates in the anti-leishmanial development pipeline (www.dndi.org).

The parts played by pharmacokinetics and medicinal chemistry have also lead to improved design of compounds able to target pathogens in infected tissues, for example in the CNS (Wring et al. 2014) and the macrophage (Rajendran et al. 2010), tissues that are relevant to the distribution of anti-trypanosomal and anti-leishmanial agents. In addition, the past decade has seen the integration of pharmacokinetics (PK), pharmacodynamics (PD) and physiology-based (PB) modelling, as illustrated in recent reviews on PK PD analysis (Nielsen and Friberg, 2013) and PB PK analysis (Edginton et al. 2006), into drug design and the prediction of appropriate dosing of novel and current anti-infective drugs. The importance of PK PD analysis has been well demonstrated for drug design for *Mycobacterium tuberculosis* (Davies and Nuermberger, 2008; Dartois, 2014), a pathogen that occupys a similar intracellular site as *Leishmania*. Another recent approach, using small molecules, has been to target host factors/receptors and nutrient sources, as considered for *Mycobacterium tuberculosis* (Zumla et al. 2014; Guler and Brombacher, 2015), and in a more limited way for leishmaniasis, for example with simvastatin (Parihar et al. 2016). Modulation of the host's immune response has been a longer term goal, with some small molecules showing activity through known targets, for example, imiquimod (Buates et al. 1999) and tucaresol (Smith et al. 2000).

Leishmania, physiological barriers and drug distribution

There is nothing new about the concept of selectivity and drug distribution as the basis for the design, discovery and development of anti-infective drugs. Over the past century our work has been framed by the likes of Paul Ehrlich (1913), "…we may speak of magic bullets which aim exclusively at the dangerous intruding parasites, strangers to the organism, but do not touch the organism, itself and its cells…". Adrien Alberts (1985) also described the challenges of achieving drug selective toxicity, comparing them at three levels – whole body

distribution, biochemistry and cytology, while linking the physicochemical properties of compounds/drugs to pharmacodynamic effects.

At the start of the drug discovery process it is essential to consider both (i) the target product profile (TPP) of the drug needed (see for example, www.dndi.org/diseases-projects/target-product-profiles) and (ii) the required distribution and pharmacokinetics of novel compounds to infected tissues. These considerations have to be integrated into the discovery and development of new treatments for both VL and CL. The design of oral drugs or topical formulations for the treatment of VL and/or CL has to account for the number of physiological barriers that the anti-leishmanial molecule meets before reaching the molecular target. Both the series of membrane barriers and the different protein binding properties in plasma and tissues (Figure 1), are challenges to be met before the novel compound reaches the macrophage host cell in which the *Leishmania* amastigote survives and multiplies (Figure 2). For CL drugs, there are the additional barriers of extravasation and the interstitial fluid compartment, factors often not considered in this complex design. These challenges are not exclusive to those working on drug R & D for *Leishmania*; similar problems arise for intracellular bacteria, in particular *Mycobacteria*, and we can learn much from that research (Dartois, 2014).

So, how can our knowledge of tissue and cellular distribution be exploited to improve drug design ? How can our understanding of membrane barriers and protein binding improve our ability to ensure drugs reach the sites where they are needed ? How can formulations and drug delivery be best exploited for this purpose ?

Selectivity and Pharmacodynamics

The initial identification of hit and lead compounds is normally determined in *in vitro* assays. Criteria for *in vitro* assays to measure killing of the intracellular *Leishmania* amastigote, were listed several decades ago (Croft. 1986); this list needs to be updated to include (i) the rate of kill, which has been shown to be important for other protozoa (Sanz et al., 2012), (ii) the type of macrophage used (see Seifert et al. 2010 below), and (iii) a panel of recent clinical isolates, as there is known strain/species variation in drug sensitivity (Croft et al. 2006). The standard phrase to introduce the *Leishmania* parasite in many reviews is "*Leishmania* survive and multiply in host macrophages". Leaving aside immunological issues of macrophage activation (Kaye and Scott. 2011), measurements of drug or NCE effects should relate to potency against a dividing population of amastigotes in a defined macrophage population. However, there are four possible states for an amastigote in the macrophage (Figure 3): stasis, division, death, or escape. These issues, are also a major concern for *M. tuberculosis* researchers, but have only recently been investigated for *Leishmania*. *In vitro* studies have given some measures of rate of division through ^{3}H-thymidine uptake (Sifontes and Croft, unpublished) and the bromodeoxyuridine analogue (EDU) (Tegazinni et al. 2016), both methods indicating incorporation these indicator of nucleic acid synthesis into about 50% of *L. donovani* amastigotes in macrophage assays. In the steps from *in vitro* assay to rodent model and from rodent model to human, it is essential to know the "predictivity" of

the model. So, are *in vitro* assays with rapid rates of division more predictive of activity in the rodent model than those with slow rates of division? Some *in vivo* studies suggest that the answer is possibly not. Recent studies on *L. major* and *L. mexicana* amastigotes in mouse models have shown that (i) *L. mexicana* amastigote division rate is slow (12 days) and sub-populations develop that are either semi-quiescent or fast-growing state (Kloehn et al. 2015), and (ii) *L. major* amastigotes in lesions similarly show fast and slow replicating populations (Mandell and Beverley, 2017). Similar questions must now be asked about *L. donovani* liver and spleen infections in mouse and hamster models to understand the relevance and predictivity of our current models. This underlines the frailty of the PD parameters currently applied in PK PD analysis for *Leishmania* models. It would be a great benefit to *Leishmania* research to be able to tranfect with reporter genes that reflect *in vivo* growth rates. In studies on *Salmonella* different bacteria cell division rates in different tissues were shown to correlate with different levels of killing by antibiotics (Claudi et al. 2014).

In addition to the above concerns about "predictivity", most amastigote-macrophage *in vitro* assays, the current workhorse of anti-leishmanial drug discovery, over the past three decades, have used either macrophage cell lines (eg THP-1 cells) or primary isolated rodent macrophages (De Rycker et al. 2013; Neal and Croft, 1984). These have on the whole have been useful in hit and lead identification. Whether there are more appropriate assays for further evaluation and lead identification has been questioned. *Ex vivo* models with splenic and lymph node tissue have been used in drug assays (Osorio et al. 2011; Peniche et al. 2014) to try to more closely match host physiological conditions. Recently we have developed a medium perfusion/flow system, using interstitial flow rates, with *L. major* infected macrophages. This a shift in the dose response curves of several standard anti-leishmanial drugs in flow vs. static systems (O'Keeffe, Croft, unpublished), where EC_{50} points were similar but EC_{90} points were significantly lower in the flow model.

Macrophages

The host macrophage is a central consideration in drug design and targeting in addition to it's role *in vitro* drug assays, from monocytic cell lines (such as THP-1, J774 lines) used in HTS, to murine peritoneal or bone marrow macrophages used in evaluation and mechanistic studies. The role of the macrophage in the immune response, pathogenesis, and parasite invasion and survival in the phagosomal compartment has been well described elsewhere (Kaye and Scott, 2011). One "macrophage" factor that is important in therapy and certainly in deriving PD parameters, is the heterogeneity of the macrophage cell population (Gordon et al. 2014) with different cell types having different functions and properties in different tissues. Recent *in vitro* studies with *L. donovani* in amastigote macrophage assays has shown that significant differences in amastigote drug susceptibility are macrophage-type dependent (Seifert et al. 2010; Konouridou et al. 2017). This is not just an issue for standardisation of assays, it is also important in understanding differences in host cell drug accumulation and role of transporters, as well as drug metabolism, for example pentavalent antimonials are metabolised by macrophages (Frezard et al. 2009) and their activity altered through macrophage activation (Murray et al. 1988). The changes in macrophage

metabolism with *Leishmania* infection could also impact drug activity and be different between macrophage populations, as has been shown for *M. tuberculosis* using RNA-seq analysis (Andreu et al., 2017). Connections between drug uptake to cell type, infection, and activation status have long been defined for antibacterials (Carlier et al. 1990). With new sensitive methods to measure anti-leishmanial drug accumulation into immune cells (Kip et al. 2015) further studies to define *Leishmania* – host cell – drug interactions are needed.

There is also opportunity to exploit the physicochemical properties of the phagolysosomal compartment, by manipulating conditions to improve drug activity, as exemplified by doxycline treatment of *Coxiella* infection (Maurin et al. 1992). The relationship between the pH of the phagolysosomal vacuole and amastigote survival is well established and pH manipulation with basic drugs, such as chloroquine, leads to parasite death. We have recently shown that in combination with chloroquine the activity of paromomycin can be significantly enhanced against *L. major* and *L. mexicana* amastigotes *in vitro* in mouse peritoneal macrophages (Wijnant et al. 2017a). The acidic environment of the lysosomal compartment of cells has been studied in relation to the accumulation of charged basic molecules, with dicationic molecules such as azithromycin having high accumulation levels (see Bambeke et al. 2006). The potential of for concentration and trapping through protonation of novel molecules was exquisitely exploited by Rabinovitch et al (1986) using amino acid esters to kill *L. amazonensis* amastigotes in mouse peritoneal macrophages. Regulation of the phagosome compartment occurs through a number of membrane enzymes and transporters, including the well-characterised vacuolar proton-ATPase (v-ATPase) involved in vacuole acidification and biogenesis (Vinet et al. 2011) and the cation transporter Nramp 1 (Jabado et al. 2000). Vacuolar enzyme and pH manipulation is being exploited in anti-cancer therapies, where the actions of known inhibitors synergize with standard drugs, with an impact on chemosensitization and reversal of chemoresistance (Swietach et al., 2012). This is an area little studied in relation to *Leishmania* ; one report has shown naloxonazine can upregulate V-ATPase in *L. donovani* infected THP-1 cells (De Muylder et al., 2016). Outside the phagolysosomal vacuole, the metabolic changes seen in macrophage biology and the numerous pathways altered by *Leishmania* infection, from iron to cholesterol and beyond, has been recently reviewed (Duque and Descoteaux, 2015).

Finally, the macrophage cell surface has several defined receptors that have been used in drug targeting to these host cells. 25 years ago Negre et al. (1992), showed that allopurinol riboside linked to mannosylated - poly-L-lysine as the carrier molecule to target the mannose receptor, improved drug activity 50-fold in an *in vitro* macrophage model. There have been several other publications describing tagged liposomes or nanoparticles in experimental models to target and increase drug accumulation by infected macrophages. However, given the added complexity of the synthesis, costs, stability and pharmacokinetics, it is unlikely that this approach will lead to new treatments for leishmaniasis.

Pharmacokinetics and Predictive models

As part of the effort to reduce the attrition rates that occur at each stage of the drug R & D process - from *in vitro* to animal models, from animal models to clinical candidate, and then in clinical trials - major efforts have been made to develop more predictive models to advance optimised novel compounds at each stage (for pharmacodynamics see above) and their behaviour in animal models and humans i.e., pharmacokinetics. PKs normally encompass the properties of a novel compound/drug of absorption, distribution, metabolism, and excretion. The anti-infective drug researcher also needs to ensure appropriate distribution of the compound to the infected tissue in the host, as well as retention of the compound within the infected tissue /cells for a period to give sufficient exposure to significantly reduce the parasite load. Over the past two decades there has been an increasing focus on the interrelationship between PDs and PKs, including tissues other than just plasma in the analysis. The PK PD paradigms were first outlined for anti-bacterials (Craig, 1998) with an initial focus on how this information, which often focuses on defining drug activities in terms of either concentration dependent rate of killing or time dependent rate of killing, can be used to help determine effective dose regimens. For anti-leishmanials PK PD analysis has helped to re-define miltefosine dosing in children for both VL and CL (Dorlo et al. 2012; Castro et al. 2017). However, for most anti-leishmanial drugs we have little data on their time-dependent or concentration-dependent activities, nor how this data can be used to optimise dose regimes. Analytical and mathematical tools are available to simulate PK/PD relationships of anti-leishmanials when there is sufficient and appropriate data. One such system has been developed for anti-malarial pre-clinical development, which has helped to identify the properties of compounds important for clinical performance and, importantly, partner drugs for combination therapy (Patel et al. 2013; Alijayyoussi et al. 2016).

This analysis of the PK PD relationship has also become central to drug development. A Pfizer team analysed the role of fundamental PK PD as part of a process to improve the survival rate of compounds in clinical trials i.e., what predictive indicators are of most importance to reduce attrition. They defined “three pillars of survival” for a novel compound as (i) exposure at the target site of action over a desired period of time, (ii) binding to the pharmacological target as expected for its mode of action, and (iii) expression of pharmacological activity commensurate with the demonstrated target exposure and target binding (Morgan et al. 2012). An important element of PK PD analysis is that *in vivo* only the free (i.e. not bound to protein) drug concentration determines activity (Smith and Kerns, 2010); hence for *Leishmania* knowledge of drug concentration in the phagosome vacuole is of importance but so far undetermined. For leishmaniasis, we have limited retrospective data on miltefosine, as summarised by Dorlo et al. (2012). More research has been reported on the liposomal formulation of amphotericin B, the unilamellar liposome AmBisome ™. We have shown that in a BALB/c mouse model (with data from liver, spleen, and plasma) that following iv dosing of AmBisome parasites in the liver are killed quicker and more effectively in those in the spleen (Voak et al. 2017). This can be explained by more extensive drug accumulation by the liver than spleen and the different drug kinetics between the two organs, although drug accumulation in these target organs was higher during the early stages of infection than later stages. Earlier studies with AmBisome in mice showed lower amphotericin B accumulation in infected than uninfected mice and also that the formulation was less effective in later stage than early stage infection (Gerskovich et al.,

2010; Mullen et al. ,1998). The effectiveness of drugs in different tissues and distribution of drug in the tissues needs to be considered alongside the changes that occur in liver and spleen structure and function during early and late stages on infection (Yurdakul et al., 2011; Kaye and Beattie, 2016). Recent studies on *M. tuberculosis* drugs to study their spatial distribution in lung lesions used MALDI mass spectrometry imaging to reveal which TB drugs penetrated the lesions (Prideaux et al, 2015); this is an approach which should be applied to help us understand the tissue distribution of anti-leishmanials.

The use of transfected *Leishmania* parasites with either fluorescent or bioluminescent properties have been used in studies on infection, pathology and chemotherapy over the past decade. But in terms of analysing key properties of anti-leishmanial drug action, these methods are only now starting to be fully exploited, in particular in rodent models of several species causing cutaneous leishmaniasis where drug efficacy and relapse have been measured (Caridha et al. 2017; Coelho et al. 2016). However, with good signal and high resolution imaging it will be important to improve the methodologies to measure (i) dose response effect, (ii) *in vivo* rate of kill and (iii) any differences in drug efficacy between sites of infection.

Drug combinations, which are present co-administrations for leishmaniasis really co-administrations, have proved to be clinically advantageous in the treatment of VL (Sundar et al., 2011). Current combinations of standard anti-leishmanial drugs are based solely on doses used in monotherapies. As novel oral compounds are developed over the next 5 years and genuine combinations are discussed, then drug combinations based upon knowledge of PK PD components of partner drugs and their interactions will be needed, as shown for antimalarials (Hastings and Hodel, 2016). We also know that major challenges for treatment are VL – HIV co-infections (van Griensven et al. 2014). There have been some *in vitro* studies on efficacy of combined anti-retroviral/anti-leishmanial drug interactions (Costa et al. 2016) and indication of anti-leishmanial activity of HIV-protease inhibitors (van Griensven et al. 2013). However, there have been few studies where a rational approach to fully understand interactions between anti-leishmanial and anti-retroviral drugs and how this knowledge could be used to design more effective treatments. In contrast to tuberculosis and malaria where drug-drug interactions have been well characterised (see University of Liverpool University UK website www.hiv-druginteractions.org) there is limited analysis on anti-leishmanial drugs.

The collection of more PD data (from HCS screens) and PK data with the need to include specific compartments for tissues infected and uninfected, plus the over-riding need to integrate all to inform dosing of the novel drug in humans, has re-focused need for the application of computational, modelling and systems biology (van der Greef and McBurney, 2005; Zhao and Iyengar, 2012). Recent mathematical model of anti-malarials has focused on simulations of PK/PD to predict clinical activity of new compounds (Aljayyoussi et al., 2016). For leishmaniasis, a disease caused by a parasite that survives, multiplies and subverts the immune responses of the host macrophage (Kaye and Scott, 2011), the elements of immunity and immunopathology also have to be built into any predictive model. A computational Petri net model of *L. donovani* infection and granulomas in mouse

liver (Moore et al., 2013; Albergante et al., 2013) illustrates another approach to disease simulation, an approach that is being further exploited to understand the relationship between the immune response and the activity and the PKs of anti-leishmanial drugs (www.crackit.org.uk/multiscale-model-minimise-animal-usage-leishmaniasis-drug-development and www.leishsim.org) .

CL, skin and topical formulations

Considering the variety of clinical manifestations and the impact of cutaneous leishmaniasis, there is a notable absence of drugs and treatments that are clinically effective (Gonzalez et al., 2008; 2009); this is an area of research neglect. This does not only apply to the classical forms of CL; there is also a need for improved treatments offering shorter courses, and less toxic drugs for post kala-azar dermal leishmaniasis (PKDL), as cases are infective to sandflies (Hirve et al., 2016) and PKDL patients act as a human reservoir for transmission and as such ARE a threat to elimination and control programmes.

Most research on CL has been focussed on immunological responses to CL in mice and humans (Kaye, Scott, 2011; Scott, Novais 2016), leading to paradigms on T-cell responses, which in conjunction with developments in knowledge on skin immunity and inflammation (see Pasparakis et al. 2014) has provided some new understanding of the pathogenesis of CL. There have been several studies over the past decade showing how this understanding of skin immune response can be exploited for treatment, with a good example being the use of imiquimod in mice and humans (Buates et al., 1999; Cesar Miranda-Verastegui, M. et al., 2009). More recently other approaches to treatment have resulted from long term human CL research in South America. Novais et al (2017) showed that NLRP3 inflammasome is activated by CD8+ T cell-mediated cytotoxicity and drives disease progression. This led to experimental studies in mice using a number of small molecule inhibitors of the inflammasome, for example, MCC950 and the diabetes drug glyburide. They showed that treatment with compounds that inhibit NLRP3 inflammasome activation, MCC950 or glyburide, failed to develop the severe disease seen in untreated mice.

Apart from the immunotherapy approaches, the design and delivery of small molecules after oral administration to the skin has to be considered in the context of vascularisation (drug gradient and distance between blood vessel and site), an interstitial fluid compartment, the impact of inflammation on drug accumulation and extra-vasation, blood flow rate (slow) and local oxygen tension (low). Improved understanding of pharmacokinetics of anti-leishmanial drugs in the skin has come from clinical studies where Dorlo and colleagues provided data on both PK and PD in human CL (*L. major)* patients treated with miltefosine (Dorlo et al., 2008) although establishing the full relationship between exposure and response has yet to be determined. We are now also beginning to understand how local inflammation at the CL site of infection can lead to specific accumulation of some drugs, for example liposomal amphotericin B, to improve cure (Wijnant et al., 2017b).

A critical decision in the development pathway for CL treatment is whether to choose systemic or topical administration. Topical formulations have been used for the treatment of CL since the 1920s when stibosan (an early pentavalent antimonial) ointment was used to

treat “oriental sore”. We have come a long way since 1935 when the use of an ointment consisting of “1 part pulverised vegetable charcoal and 9 parts of concentrated sulphuric acid” was described. However, the full exploitation of pharmaceutics and knowledge of skin, from utilisation of knowledge of skin physiology and PB PK models, alteration of vasculature, role of protein binding and other factors (Jepps et al. 2013), including lymphatic flow to deeper layers (Dancik et al. 2011), are only now being applied to the development of new treatments for CL. The renaissance in the topical approach was led by El-On et al. (1984) with paromomycin, using an irritant and transdermal enhancing agent (methyl benzethonium chloride) to increase drug permeation by pore formation, the basis for the product Leishcutan ®(Teva, Israel). Another formulation of topical paromomycin, containing 15% paromomycin–0.5% gentamicin and several excipients (called WR 279,396) with known absorption and skin PK (Ravis et al. 2013) when applied with an occlusion, has successfully completed phase 3 trials (Ben Salah et al. 2013).

Focus on potency, permeation, and distribution (Jepps et al. 2013) is important for both formulation design and the selection of appropriate compounds with both high potency and, through their chemical properties, dermal distribution. As part of our strategy, we : (i) identify novel compounds that work against a panel of clinical isolates of the 15 species, as *Leishmania* species that cause CL vary significantly in their drug susceptibility (Escobar et al.,2002; Croft et al. 2006), (ii) select active compounds with appropriate medicinal chemistry, toxicity and ADME (absorption, distribution, metabolism, excretion) properties, (iii) test in mouse models of infection (oral and systemic administration), (iv) optimise for compound structure and formulations in relation to skin distribution, (v) decide whether topical administration is appropriate and further optimise the formulations using both mouse and human skin in permeation studies, and (vi) ensure that treatment is effective against early stage infections with intact skin (prior to ulceration) as the aim to develop a treatment effective before the patient has developed into a large disfiguring ulcer.

When considering the pharmacokinetics of drugs for CL, it is important to remember that the *Leishmania* are in macrophages in the skin dermis, and that the infection is not superficial like many bacterial or fungal infections. Within the dermis at the site of infection, there is well characterised inflammation and and granuloma development (Scott, Novais, 2016) and amastigotes in macrophages. This is found in both the nodule that precedes ulceration or later in the dermal rim around the ulcer. The aim for systemic formulation is penetration from the vasculature via the interstitial fluid and distribution to the inflammatory site of infection. However, for a topical formulation the aims are permeation of the skin barriers and then distribution to the site of infection. In both cases exposure following distribution and residence of the compound at the site of infection has to be optimised. Some of the practices of pharmaceutical scientists working on skin for cosmetics and other purposes have been adopted for CL studies over the past decade. Using methodologies like the Franz cell, it is possible to measure the rate of diffusion of anti-leishmanial drugs across skin (of animal models and humans) alone and in different formulations, as shown for buparvaquone where the most effective topical formulation *in vivo* proved to be the one that crossed the skin most slowly in the Franz cell model (Garnier et al. 2007a,b). Recently this work has been extended to include *Leishmania* infected skin.

Permeation markers, for example caffeine and ibuprofen, as well as some standard anti-leishmanial drugs have been shown to have different *in vitro* permeation properties through normal mouse skin, compared with mouse skin removed from a nodule of infection (Van Boxclaer et al. 2016a). Drugs permeate significantly faster through skin taken from the site of infection, possibly due to oedema and the different immunological profile at this site of inflammation. As more extensive exposure in the dermis is critical to formulation design, the permeation properties of formulation excipients alone and together need to be explored. A re-examination of topical formulations of the anti-leishmanial drug miltefosine, using *in vitro* and *in vivo* models already mentioned, and a range of formulations in which the partition of miltefosine was characterised, was unable to identify a formulation with good permeation and efficacy (Van Bocxlaer et al. 2016b).

Conclusions for Leishmaniasis drug R & D

There have been several reviews that represent the drug R & D process as a linear diagram from discovery to clinical trial. However, drug R & D is a multi-disciplinary iterative process with many decision points, and the involvement of several teams across disciplines (Baxter et al. (2013). The parasitologist has key roles within this complex picture and an awareness of the comprehensive list of detailed information that s/he should aim to provide as part of a drug research team – ranging from work on enzyme targets to pharmacokinetics. Although the concept of the "minimum information about a bioactive entity (MIABE)" (Orchard et al., 2011) was established to provide guidance for what and how results should be reported, their review also provides a fundamental list of research information that needs to be gleaned from studies. In the specific area of leishmaniasis and *Leishmania*, where there are a large variety of assays and models involving different species, strains and stages of the *Leishmania* parasite, different host cells and different mammalian hosts, it is hardly surprising that there can be significant differences in data obtained between laboratories resulting in reports of irreproducibility of compound activities. In addition to basic precepts, such inclusion of controls, Figure 4 is an attempt to summarise the main PD and PK related factors that must be considered when collecting data during drug discovery and early pre-clinical studies for a novel anti-leishmanial compound. Although there are benefits for standardisation, a process necessary when determining drug sensitivity of clinical isolates (Hendrickx et al. 2017), it is hardly feasible in the drug R & D process. But it is feasible for all those concerned to provide the levels of information sought and provided (Orchard et al. 2011) so that data can be interpreted by all those interested in playing a role in the development of the next drug and treatment for leishmaniasis.

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