ELSEVIER

Walking a tightrope: drug discovery in visceral leishmaniasis

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The current commitment of the pharma industry, nongovernmental organizations and academia to find better treatments against neglected tropical diseases should end decades of challenge caused by these global scourges. The initial result of these efforts has been the introduction of enhanced combinations of drugs, currently in clinical use, or formulations thereof. Phenotypic screening based on intracellular parasite infections has been revealed as the first key tool of antileishmanial drug discovery, because most first-in-class drugs entering Phase I trials were discovered this way. The professional commitment among stakeholders has enabled the availability of a plethora of new chemical entities that fit the target product profile for these diseases. However, the rate of hit discovery in leishmaniasis is far behind that for other neglected diseases. This review defends the need to develop new screening methods that consider the part played not only by intracellular parasites but also by the host's immune system to generate disease-relevant assays and improve clinical outcomes.

Introduction

Leishmaniasis (see Glossary) is a neglected tropical disease (NTD), endemic in 98 countries. Currently, 12 million people are affected with leishmaniasis, >350 million people are at risk of contracting the infection and over one million new cases are reported each year. This parasitic disease has four main clinical syndromes: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral or Kala-azar leishmaniasis (VL) and post Kala-azar dermal leishmaniasis (PKDL). VL is ranked second in mortality and fourth in morbidity among NTDs, with 20 000–40 000 deaths annually. The majority of cases (90%) are reported in three endemic foci: (i) India; (ii) East Africa; and (iii) Brazil [1]. In the absence of vaccines, chemotherapy and vector control are the tools for disease management [2].

In 2005, the Indian Subcontinent, where the disease is caused by *Leishmania donovani* and has anthroponotic transmission, launched an initiative to eliminate the disease by 2017 [3]. In 2014, Bhutan and Thailand joined the initiative and the goal was set for 2020 [4], although this has since been delayed [5]. The potential role of asymptomatically infected humans and animals, along with the important reservoir of infection that can represent PKDL patients, are some reasons raised to explain this delay [5]. Recently, new models of transmission, including not only active VL but also asymptomatic and PKDL patients, concluded that the current policy recommendations by WHO, which are based on the combination of indoor spraying of insecticide and the reduction of onset-to-treatment times, would be sufficient to achieve the elimination target in all settings [6]. Treatments are mainly based on a single dose of liposomal amphotericin B (LAMB) [7] but its poor stability along with cold-chain issues in endemic districts imply other alternatives based on combination therapy, such as a single dose of LAMB (5 mg/kg) plus 7 days of oral miltefosine (50 mg once daily) or single dose of LAMB plus 10 days of paromomycin sulfate (15 mg/kg/day) or miltefosine (50 mg once a day for 10 days) plus paromomycin (15 mg/kg/d for 10 days) [8]. In East Africa, where AIDS is epidemic, VL with HIV co-infection has become a serious challenge [9]. The first-line treatment based on pentavalent antimonials has been replaced with the combination of sodium stibogluconate and paromomycin (SSG

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GLOSSARY

Anthroponotic transmission an infectious disease where a disease-causing agent carried by humans is transferred to other animals. It can also be defined as a human-to-human infection with no animal reservoir.
Chemogenomic library a set of small-molecule pharmacological agents. An identified hit from this library implies that the annotated target of that hit ccould be involved in modulating the observable phenotype.
High-content screening combination of automated microscopy and image analysis that allows multiparametric detection and quantification of phenotypes of interest. It allows large-scale compound screening to identify desired cellular phenotypes.
Hit a compound that kills intracellular parasites without cytotoxicity for host cells. Its activity is confirmed upon retesting.
Leishmaniasis is a group of neglected diseases caused by various species of protozoan parasites belonging to the genus Leishmania and transmitted to mammal hosts (anthroponotic and/or zoonotic) by the bite of infected female phlebotomine sandflies. WHO has catalogued >20 Leishmania species associated to the four main forms of the disease that are responsible for various clinical manifestations from self-healing ulcers to fatal outcome.
Phenotypic drug discovery an approach that uses whole-cell screening to identify effects on a target cell or pathogen without the need for understanding the underlying mode of action.
Drug repurposing new applications for approved drugs or early- or late-stage candidates with availability of pharmacokinetic and safety profiles. It represents a fast and cost-effective strategy to obtain new therapies. Amphotericin B, miltefosine and pentamidine were previously approved for other indications.
Selectivity index the ratio between the IC_{50} and CC_{50}, the half-maximal inhibitory concentration against the parasites and the half-maximal cytotoxic concentration against the host mammalian cells. It reflects the quantity of compound that is active against the pathogen but is not toxic toward the host cell.
Target-based drug discovery an approach that hypothesizes a direct link between the parasite target and the resolution of the disease.

+ PM) for 17 days, which reduces the overall cost and time of treatment [10]. Although the evidence is scarce, LAMB does not appear as efficacious in Africa [11]. Brazil represents >96% of VL reported in Latin America. It is a zoonotic disease produced by *Leishmania infantum,* with dogs being the main reservoir and children <10 years being the most affected patients [12]. A recent clinical trial in Brazil that compares amphotericin B formulated as deoxycholate or liposomal, alone and in combination with antimonials, points toward a recommendation for the use of LAMB as the first-line treatment for VL not only in Brazil but in other Latin American countries [13]. This scenario demands the challenge of urgently releasing new, safer and better drugs, with new mechanisms of action, at cost-efficient development and that are patient-friendly use (ideally oral), despite the tough financial landscape associated with these diseases.

Opportunities and challenges from phenotypic screening assays

The discovery of new drugs is a long, difficult and expensive process, estimated to last 10–17 years and cost ~US$1.8 billion, with a success rate of ~10% [14]. How can this process be expedited?

Drug repurposing

Drug repurposing is the preferred strategy to accelerate drug discovery in a field with scarce funds and urgent need to obtain a cost-effective final product. This is mirrored by the compounds that are currently used in clinical treatment (amphotericin B, miltefosine, PM). For a recent review about repurposing in leishmaniasis we refer readers to [15]. It is worth bearing in mind that the drug repurposing strategy will accelerate the overall process and reduce the cost of drug discovery as long as the chemical libraries are well annotated, and the pharmacokinetic and safety data of the compounds are available to researchers. The main challenge would reside in establishing the connection between the old drug and the new target [16].

Chemical space and unknown targets

Before screening strategies are decided, two issues might be crucial when starting a drug discovery campaign: the chemical space and the potential known and unknown targets. Chemical space is estimated to be 10^{60} compounds; whereas the number of synthesized molecules currently deposited in PubChem is 50 orders of magnitude lower [17]. At this point, the identification of biologically relevant chemical space is crucial. Two approaches have been proposed: (i) the use of algorithms trained to seek bioactivity through basic chemical rules [18]; (ii) the comparison to compounds that share the same pharmacophore of natural products based on the principle that they have been evolutionarily selected to bind proteins [19]. According to this hypothesis, Gerry and co-workers, taking advantage of the rapid progress in modern synthetic chemistry, suggest the inclusion of small molecules with structural features that are common in natural products but that are poorly represented in screening collections, either from commercial or pharma-industry origin [20]. Regarding the target space, a recent piece of work by Cravatt and co-workers, who performed a phenotypic screening assay using fragment libraries, showed that only 17% of the human proteome has known ligands [21]. For *Trypanosomatida*, the scenario can be even worse given the large number of genes encoding hypothetical and putative proteins with unknown function in their genome databases.

Target-based and phenotypic screening assays are complementary

The initial steps in drug discovery include the identification of active compounds (hits), which are subsequently optimized in an iterative process until reaching the clinical candidate. The choice between target-based drug discovery (TDD) and phenotype-based drug discovery (PDD) is the starting point of any antileishmanial drug discovery campaign. Although based on opposed paradigms, they complement each other, thus increasing the odds of discovering and developing new drugs with new mechanisms of action. The publication of the TriTryps database created great expectations in TDD, although the lack of translation from *in vitro* results to whole-cell assays or *in vivo* activities has recommended PDD as a starting point in many screening labs.

TDD hypothesizes that a defined molecular target (protein) plays a crucial part in disease development. However, the biological pathways essential for leishmania survival and disease progression are not yet well established and, therefore, there is a lack of well-validated drug targets [22]. Field and co-workers have illus-

trated the targets against *Trypanosomatida* and their degrees of validation [23]. By contrast, PDD evaluates phenotypic changes introduced by an external agent on a cell, tissue or entire organism, regardless of its particular mechanism of action. Therefore, one of the main demands in leishmaniasis therapeutics is the identification of compounds with new mechanisms of action to provide alternative pathways and overcome resistance to current drugs. The preclinical stages of PDD programs require greater resources than TDD, because the inclusion of a compound into the development stages without understanding its mechanism of action involves high risk. Therefore, target identification and the understanding of the mechanism of action are not essential but helpful on the drug discovery strategy (from hit-to-lead and lead optimization stages) [24].

In antileishmanial drug discovery, automated high-content screening (HCS) has increased the throughput of intracellular assays [25–27]. It is generally accepted that the intracellular infections are better models than extracellular parasite stages in catching the essentials of disease in humans. Additionally, they can identify hits that reach the necessary intracellular concentrations to kill the parasite inside the host cell without affecting the macrophage. However, when a large number of compounds are tested, a cascade screening process is performed, which includes a primary screening campaign in free parasites complemented with a secondary screening campaign with intracellular amastigotes [28].

Selectivity and safety during phenotypic screening assays

A panel of experts from the Medicines for Malaria Venture, Drugs for Neglected Diseases Initiative and TB Alliance, together with representatives from the Bill & Melinda Gates Foundation, have established the general and specific hit and lead criteria for four neglected diseases, including VL [29] (Box 1). Potency is the first parameter obtained from screening assays, but potency alone is a false predictor for hit selection. Hit selection and prioritization can be based on two different categories. The first refers to the biological profile that includes potency, selectivity and specificity. The second category refers to physicochemical properties that includes drug pharmacological and toxicological behavior (ADMET) [30] (Fig. 1).

Selectivity and specificity after PDD approaches, with no single clue about the mechanism of action, should be assessed as early as possible through orthogonal *in vitro* assays, such as cytotoxicity in host cells (CC_{50} determination) including established human cell lines (HepG2, MCR-5, THP-1, etc.). The selectivity index informs about the comparative toxicity for the parasite over a mammalian cell line and, along with an acceptable dose–response curve, provides confidence in specific interactions and effects. Generally, it is well accepted that high-level potency is a good criterion for hit eligibility as long as safety and selectivity are guaranteed. However, because the emergence of resistance is a global issue, another filter of hit prioritization is the performance of a secondary screening of primary hits against resistant clinical isolates (if available) [31].

BOX 1

Hit and lead criteria in drug discovery for visceral leishmaniasis

Primary phenotypic-based drug discovery in *Leishmania spp* is initiated at concentrations >10 μM, which is considered an inclusive criterion. Libraries are tested and compared with a set of selected active and inactive compounds included in the screening platform. Those compounds with the best inhibition rates (e.g., 70–100%) are introduced in a second-round screening (dose–response) that includes serial dilutions to determine the potency {−log(inhibitory concentration IC_{50}[M])}. Hits should show a sigmoidal concentration–growth inhibition curve reaching a maximal 100% efficacy, with a Hill slope coefficient ideally between 0.5 and 1.8 [29]. A hit should show at least a tenfold selectivity index using a mammalian cell line. Given the risk of emergence of resistant strains, it is recommended to evaluate the potency *in vitro* of the potential hit through a panel of recent clinical isolates with variation in drug sensitivity [29].

A lead is defined as a compound with *in vitro* IC_{50} <10 μM against an intracellular parasite and selectivity index >100-fold, resulting in a >70% reduction in liver parasite burden *in vivo* after at most five doses at 50 mg/kg bodyweight administered orally once or twice per day. Finally, a promising lead should lack promiscuous bioactivity and accomplish a minimum early safety assessment including *in vitro* genotoxicity studies (Ames-test), cardiotoxicity and *in vivo* tolerability studies during the administered doses [29].

Hit expansion: lead optimization and in vitro/in vivo *pharmacology*

Besides potency and selectivity of identified hits, ADMET and physicochemical properties help to decide which hits (chemical clusters and singletons) should advance into the lead optimization phase. Progress in *in silico* and *in vitro* methods can help to improve the predictions from *in vitro* to *in vivo* experiments to prioritize the better compounds [32]. This is a three-filter selection panel based on *in silico* ADME-PK predictions [33], *in vitro* experimental characterization and, finally, *in vivo* pharmacokinetic/pharmacodynamic (PK/PD) studies. *In silico* tests can help to prioritize the selected compounds, thus minimizing time and cost efforts. Nevertheless, no hits will be discarded based on *in silico* information only. *In vitro* characterization selects those hits with desirable physicochemical properties (solubility, lipophilicity), which represent an as wide as possible biologically relevant chemical space. High-to-medium-throughput assays, such as permeability through artificial membranes (parallel artificial membrane permeability assay, PAMPA) or Caco-cultures, binding to serum proteins, cytochrome P450 inhibition and chemical stability in liver microsomes across species, provide valuable information. Finally, those hits with the most promising properties in the previous screens will move forward to the third filter, which evaluates *in vivo* PK and provides information after oral exposure [area-under-curve (AUC), maximum blood concentration (C_{max}) and half-life ($t_{1/2}$)]. Croft has recently summarized some PK/PD-related factors that must be considered when data are collected during early preclinical studies, including the replication rate of intracellular parasites, the nature of host cells, the rate of kill and differences in drug efficacy between sites of infection [34].

Establishing predictive phenotypic screening assays

Intracellular parasite screens

HCS technology has transformed the field of drug discovery during the past decade. The robotic automation of confocal microscopy aided by powerful image software has permitted the assessment of millions of small molecules under PDD conditions. This has

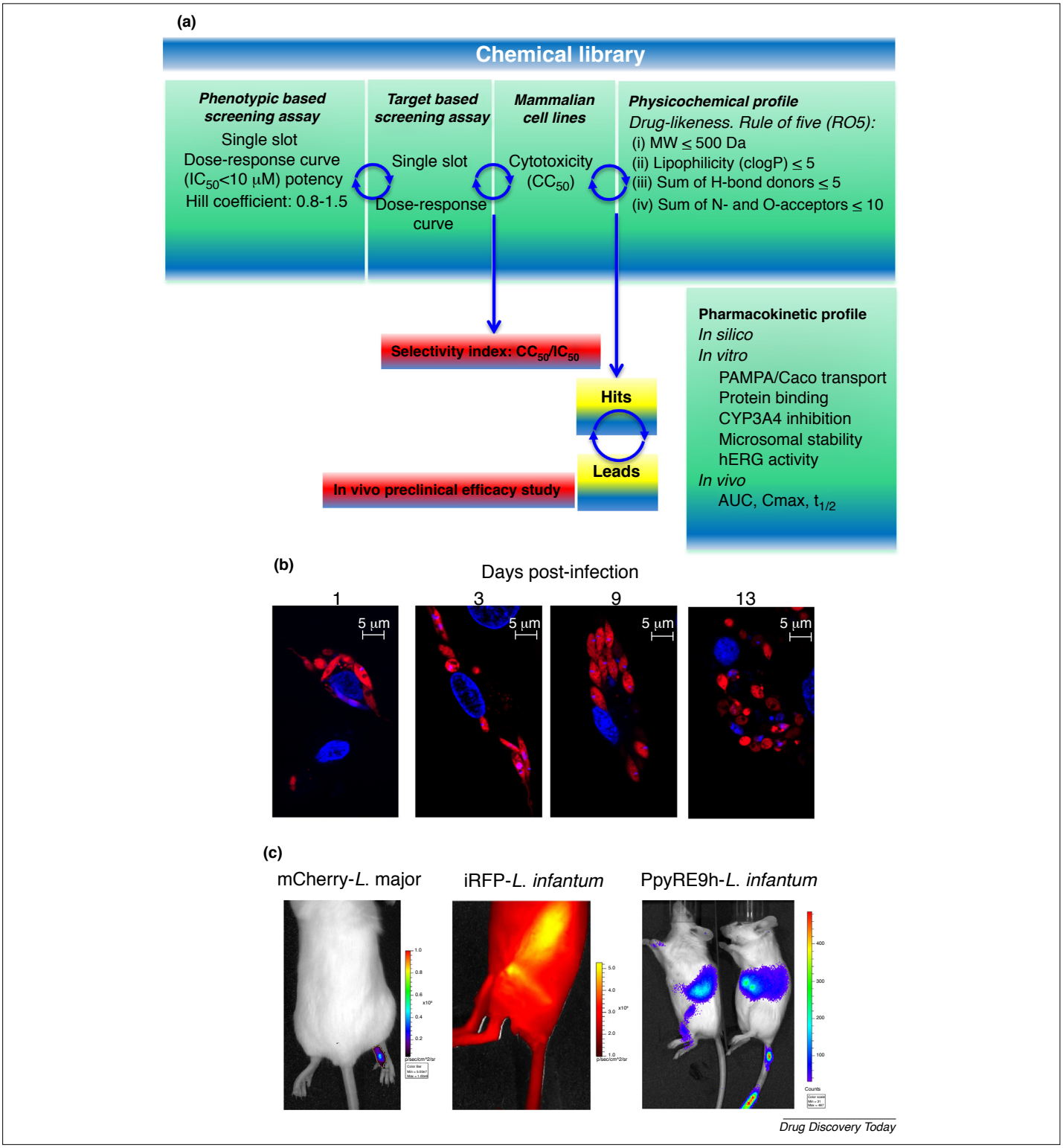

FIGURE 1

(a) Cascade screening platform for identification of hit-to-lead antileishmanial drugs. Screen assays based on phenotypic approaches performed on single slot. Starting concentration and cut-off activity is fixed in the screening campaign (e.g., concentration between 20 and 50 μM and antileishmanial activity >55%). After confirming outcomes in individual and repeated experiments, selected hits are tested in dose–response curve assays to identify those with IC_{50}s ≤10 μM against intracellular parasites. Orthogonal assays to test cytotoxicity using different cultured cell lines help to establish the selectivity index that should be tenfold for hits and 100-fold for leads. Criteria for drug-likeness can be used to prioritize hits. They are biophysical properties for oral absorption known as the Rule of Five (RO5) that establish limits in molecular weight (MW), number of H-bond donors and N- and O-acceptors and lipophilicity (logP). In addition, leads must show specific binding (dose–response curve and selectivity index), emerging SAR, pharmacokinetic properties (ADMET). First, *in silico* tools are used to explore ADMET properties. Then, high-to-medium throughput *in vitro* assays are used to characterize the selected hits. Oral absorption (PAMPA assay), distribution (plasma protein binding), metabolism (cytochrome p450 inhibition, metabolic clearance) and toxicity such as human ether-a-go-go related potassium channel binding (hERG). A third filter includes an *in vivo* ADME profile to establish area-under-curve (AUC) and C_{max} as key parameters reflecting oral

provided multiparametric information, such as the number of infected macrophages, the number of intracellular amastigotes, the toxicity for host cells and other values related to assay robustness and reproducibility [35].

PDD screens based on promastigotes and axenic amastigotes do not identify all active compounds and yield a large number of false-positive results [25–27]. The most powerful results in this regard were presented by Peña and co-workers after analyzing a diversity set of 1.8 million compounds against the three kinetoplastid parasites responsible for NTDs. Unfortunately, despite this enormous effort, no correlation was found between the antileishmanial potency determined in amastigotes grown under axenic conditions and intramacrophagic *L. donovani* assays [36]. In addition, very little is known about how *in vitro* potency correlates with *in vivo* activity in leishmaniasis, together with the lack of available PK/PD data. Ortiz and co-workers performed a comparative efficacy study *in vivo* with *Leishmania mexicana* after *in vitro/in vivo* PK studies. Despite the fact two selected hits were identified through promastigotes and intramacrophagic screens and showed good PK properties, they were unable to control the lesions produced by the parasite [28]. Eren *et al.* used a library of FDA-approved compounds and detected disulfiram as a potential hit against *Leishmania guyanensis*. However, despite the potency of this compound being within the nanomolar range, the *in vivo* efficacy was low [37]. Partly because of the paucity of available data, it is difficult to show how valid current antileishmanial phenotypic screening assays are or their predictive value. By contrast, it is worth highlighting that most of the compounds currently filling the pipelines for leishmaniasis were identified in PDD assays [38]. The input provided by organizations, such as DNDi, in collaboration with the pharma industry (e.g., Novartis and GSK, among others) has greatly improved access to PK/PD data, which has fostered a more professional drug discovery for VL, thereby increasing the number of candidates entering clinical phases. This has been corroborated over the past years by the huge increase in the new chemical entities proposed to enter clinical trials [39–44] (Fig. 2).

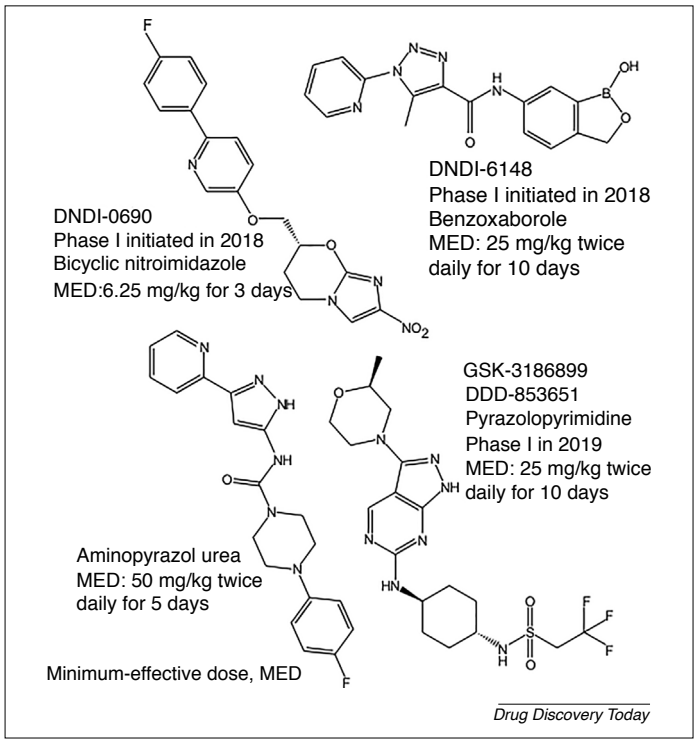

FIGURE 2

Chemical structures and key properties of some compounds entering Phase I trials. DNDI-6148 from the oxaborole class and DNDI-0690 from the nitroimidazole class have entered clinical Phase I trials. Aminopyrazoles and pyrazolopyrimidine GSK-3186899 (DDD853651) are shown.

Chain of translatability: molecular phenotyping

The question that arises in this regard is how to establish the key factors for a successful drug discovery plan using phenotypic screening. Moffat and co-workers have introduced the concept of chain of translatability as 'the presence of a shared mechanistic basis for the disease model, the assay readout and the biology of the disease in humans, as a framework for developing phenotypic screening assays with a greater likelihood of having strong predictive validity'. In the field of infectious diseases, phenotypic projects are considered to have a strong chain of translatability, because the inhibition of the pathogen replication mechanism can strongly correlate with good efficacy in preclinical models and a cure in patients. The ability to capture a relevant mechanism of action in the screening system of human leishmaniasis is of paramount importance to develop phenotypic screening assays with predictive value and translatability to humans [24].

PDD based on cultures of infected macrophages *in vitro*, although widely used at present, does not incorporate other disease cells involved in the host immune response. To decipher host–parasite interactions, different approaches have been followed. Early studies with *in vitro Leishmania chagasi* infections revealed that macrophage response was significantly regulated by the addition of autologous naive T cells. The transcription profile was downregulated in infected cultures containing macrophages alone and, surprisingly, the addition of T cells to infected macrophages resulted in an inflammatory response [45]. More recently, RNAseq-based meta-transcriptome profiling was carried out to compare the expression of *Leishmania braziliensis* amastigotes derived from patient lesions with those transcripts expressed *in vitro* in human macrophages infected with *Leishmania amazonensis* or *Leishmania major* and showed discordant results [46]. Although different *Leishmania* species were used, parasite transcripts expressed in patient lesions did not coincide with those expressed by intracellular amastigotes *in vitro*. These results open a debate about whether *in vitro* macrophage monolayers are good predictors of *in vivo* lesions at the level of gene expression and, therefore, this *in vitro* model raises questions about its ability to capture the molecular phenotype for the screening system.

exposure and required to perform early *in vivo* efficacy studies. **(b)** Infrared fluorescent *Leishmania infantum* strain (iRFP *L. infantum*) during an experimental infection of human monocytes THP-1. The infection days show the progressive transformation of metacyclic promastigotes to round amastigotes. Images were taken with a confocal Zeiss microscope. **(c)** Transgenic strains allow appraisal of infection in real-time. mCherry *Leishmania major* Balb/c model (left panel). iRFP *L. infantum* Balb/c model (center panel). PpyRE9 *L. infantum* Balb/c model (right panel). Pictures were taken using an IVIS-Lumina fluorescent recorder device.

During the past years, there has been an increased trend toward the use of disease-relevant assays at the phenotypic screening, using primary cells, patient cells, co-cultures and 3D cultures, which is currently impossible in the NTD setting [47]. However, an alternative to explore and develop more-predictive phenotypic assays is the *ex vivo* splenic-explant [48,49]. It contains the full repertoire of infected and uninfected immune cells (co-culture model), mirroring the complex interaction between parasite and host. In addition, the co-culture shows the exhaustion conditions suffered by lymphocytes during chronic infections [50]. Thus, it provides a similar scenario to real *in vivo* infections, although soluble factors from the inflammatory milieu could be lost. The assay is performed in 384-well plates, and a single infected animal is sufficient to screen >500 compounds. Thus, its throughput can be similar to other intracellular assays. It requires the use of animals, which could represent an ethical concern. However, it provides a system where cytotoxicity can be simultaneously measured in primary cell cultures. Its odds of successful translatability from *in vitro* assays to *in vivo* efficacy models and human patients still have to be proven. However, it can be a good starting point to invest more time and develop follow-up assays that help to connect the molecular phenotypes with the screening assays.

In vivo animal testing

Animal models in visceral leishmaniasis

At the preclinical stage of drug discovery, the efficacy of those hits and leads, identified *in vitro* and showing good PK/PD properties, has to be verified before first-in-human Phase I study, through *in vivo* tests in animal models. The best preclinical models in drug discovery are those that closely recreate the human disease in relation to its etiology, pathophysiology, symptomatology and response to therapeutic agents, gaining insights about the efficacy and safety of the treatment. Their predictive value increases as long as they have greater ability to simulate the human disease.

During past decades, many experimental models have been developed, although none of them accurately reproduces human VL. Among them, rodents such as BALB/c mice and Syrian golden hamsters (primary tests) are preferred for initial preclinical stages, because they facilitate pre-formulation design, PK/PD and submission of novel chemical entities to regulatory agencies. In addition, these models are cheaper, easy to handle and, crucially, they require a lower amount of drug [50]. However, there is certain controversy about the best animal model to be used in experimental VL. The hamster model more strictly reproduces the progression of the human visceral disease (face validity), whereas the mouse model is more suitable for acute and chronic subclinical infections [51]. By contrast, using high infective doses of *L. infantum* and *L. donovani*, mouse spleen shows hallmarks of progressive human disease [52]. The results obtained with these models are influenced by several experimental parameters, including the genetic background, parasite genotype, inoculation route or infection site, parasite dose and the composition of the phlebotomine saliva [53].

Real-time in vivo *imaging in experimental visceral leishmaniasis*

To advance faster in the discovery of novel antileishmanial drugs, where the death of parasites closely corresponds with the therapeutic effect of the drug, researchers have developed animal models based on real-time *in vivo* imaging. These approaches use transgenic parasites transfected with foreign genes encoding bioluminescent and fluorescent reporters, allowing the precise detection of parasite burden in a noninvasive animal model using sensitive charge-coupled cameras (CCD) [54]. Early real-time *in vivo* approaches were performed in a CL model, because the parasites are located mainly in shallow locations such as the footpad. For instance, a luciferase-expressing *L. major* strain was used to assess *in vivo* the aminoglycoside topical formulation (WR279396) [55], and transgenic green fluorescent protein (GFP)-labeled *L. amazonensis* and mCherry-expressing *L. major* parasites were used to evaluate the efficacy of several vaccine candidates [56–59].

In the case of VL, where the tissues involved have deep locations (liver, spleen and bone marrow) only bioluminescent (no fluorescent) models have enough sensitivity to visualize the infection in mice [60] and hamsters [61]. More recently, the red-shifted firefly luciferase PpyREH9 has been used in combination with (or fused to) fluorescent proteins to assess the efficacy of new compounds [62,63]. By using modified parasites expressing bioluminescent and fluorescent proteins, parasite burden can be easily detected *in vivo* and automatically quantified *ex vivo* using microplate assays [64]. Real-time *in vivo* imaging might also help understand the physical and temporal dynamics of the parasite at the tissue or organ level in the mammalian host. The current weaknesses of this technique include the need for light-emitting substrates to detect the bioluminescent activity of reporters, although future and more-sensitive near-infrared fluorescent probes could be of help in this regard.

Concluding remarks

In summary, antileishmanial drug discovery is still a challenge, despite the great efforts made during recent years. This challenge shares, with any drug discovery program, key factors such as chemical diversity in libraries, availability to PK and safety data. Some other factors are more specific to the disease, such as the low number of validated targets or the lack of connection between molecular phenotypes and the screening tests. The new antileishmanial candidates identified from phenotypic screening, which are based on intracellular assays, highlight the power of these approaches. However, many drug candidates ready to enter first-in-human trials were initially identified in other programs against tuberculosis and African trypanosomiasis. Thus, it is difficult to state whether current phenotypic assays would be effective for the identification of compounds from unbiased libraries.

The complex host–parasite interaction is not reproduced in a monolayer culture, and many of the early *in vitro* experiments are discordant with more-recent results obtained from patient lesions. High-content imaging has boosted drug discovery, although disease-relevant assays including primary patient cells or 3D systems used in other discovery programs are unapproachable in NTDs. Therefore, it is necessary to make more effort developing phenotypic screening assays with disease-relevant models and to include *in vivo* efficacy studies as early as possible to achieve better predictive values.

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