

Tools for Antileishmanial Drug Discovery and Drug Development

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Efforts for the development of new therapeutics, essential for the control of Leishmaniasis is on for decades. Current treatment is based on chemotherapy, which relies on handful of drugs with serious limitations such as high cost, toxicity, difficult route of administration, lack of efficacy in endemic areas and drug resistance. Pentavalent antimonials are the mainstay of antileishmanial therapy for over 70 years with second line drugs, Amphotericin B and Pentamidine used in case of antimonial failure. Alarming increase in drug resistance and the absence of an effective vaccine has made the development of effective antileishmanials a prerequisite. Toxicogenomics, proteomics, metabonomics and pharmacogenomics represent the latest experimental approaches that can be combined with high throughput molecular screening of targets to provide a view of the complete biological system that is modulated by a compound. This review highlights the different approaches to antileishmanial drug discovery and identification of novel antileishmanial compounds.

Introduction

Leishmaniasis is a major health problem that affects approximately 12 million people worldwide, with 2 million new cases diagnosed every year¹. Leishmaniasis presents a broad clinical spectrum, ranging from asymptomatic and self-healing infections to those causing significant mortality². There is a dramatic increase in the number of cases of leishmaniasis which has been observed in HIV patients³.

Despite of prevalence of leishmaniasis, there are no vaccines or prophylactic drugs for any form of the disease. Current chemotherapeutic treatments rely heavily on the use of the pentavalent antimonials⁴. These compounds have serious side effects and are declining in efficacy due to chemoresistance⁵. Second-line drugs, such as pentamidine and amphotericin B, are available but they too have significant untoward effects and pharmacological liabilities. Few groups have attempted to augment the pool of available leishmanicidals by exploiting drugs approved for other diseases. To maximize effectiveness and minimize toxicity, the choice of drug dosage and duration of therapy should be individualized based on the region of disease acquisition and host factors such as immune status. Regrettably, there is a paucity of large-scale drug discovery efforts focusing on the design of new chemical entities that can treat individuals with leishmaniasis. Several new antileishmanial compounds are under development, but a promising drug which could qualify for radical cure of these infections has not been documented. Currently the discovery of compounds to combat tropical diseases is based on

several strategies which will be discussed in the present review.

Chemotherapy of Leishmaniasis

The current drug discovery scenario

Pentavalent antimonials such as sodium stibogluconate and meglumine antimoniate are the first line drugs which play a major role, but due to resistance and toxicity their use is limited. Second line drugs such as amphotericin, pentamidine, miltefosine, paramomycin (Fig. 1) although effective in treatment, possess several side effects which are enlisted in Table 1. It is obvious that new drugs or strategies must circumvent limitations such as a long-term parenteral administration, toxicity, the high cost in endemic countries, and the emergence of resistance⁶.

Drugs in development and clinical trials

The 8-aminoquinolone, sitamaquine is being developed as an alternative oral drug for the treatment of visceral leishmaniasis. An initial Phase II study resulted in a 50% cure rate⁷, whilst later studies provided more variable results, and showed toxicity that had not been apparent in previous studies^{8, 9}. The other drugs in clinical trial are the antimycotic azoles, ketoconazole, itraconazole, fluconazole, posaconazole and imiquimod (Fig. 2)¹⁰⁻¹².

Preclinical and Experimental Agents with Antileishmanial Activity

A variety of natural products possess antiparasitic activities, however, a majority of these products also have undesirable properties such as high toxicity, poor solubility, low bioavailability, low effectiveness at moderate doses and unsuitability for oral or topical application¹³. Some of the promising antileishmanial compounds are listed in Fig. 3.

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Table 1. Adverse effects of conventional therapies against leishmaniasis

S. No.	Drugs	Adverse effects
1.	Pentavalent antimonials	Arthralgia, myalgia, nausea, vomiting, headache, rash, abdominal pain, transaminase elevations, pancreatitis, anemia, leucopenia, thrombocytopenia, reversible renal insufficiency and cardiotoxicity.
2.	Amphotericin B nephrotoxicity.	Fever, malaise, nausea, vomiting, hypokalemia, rash, phlebitis, anemia, hypomagnesemia,
3.	Pentamidine	Hypoglycemia followed by diabetes mellitus, hypotension, nausea, vomiting, headache and abdominal pain.
4.	Miltefosine	Nausea, vomiting, diarrhoea.
5.	Paramomycin	Fever, swelling, rigors and vomiting.

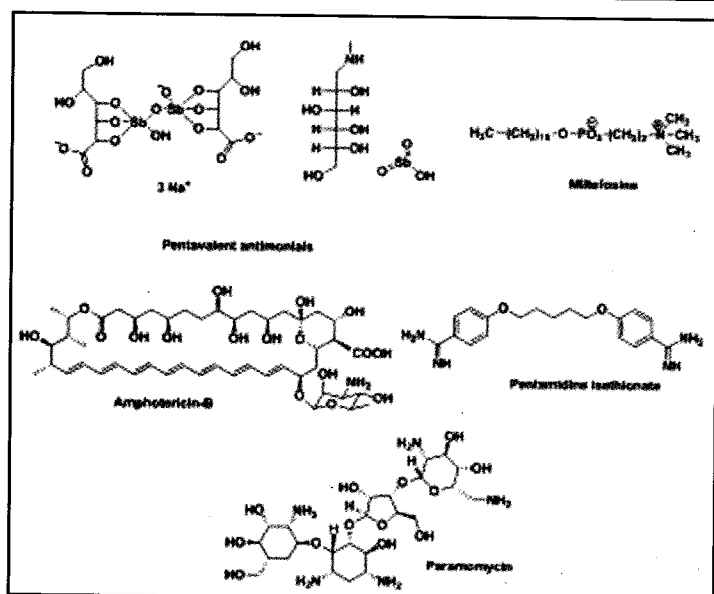


Fig. 1. Chemical structures of anti-leishmanial drugs; pentavalent antimonials, miltefosine, amphotericin B, pentamidine, paramomycin.

N-methylated linear lipopeptides, almiramides A-C¹⁴, Arylimidamides (AIAs) DB745 and DB766¹⁵, Nelfinavir, an HIV-1 protease inhibitor¹⁶, the natural curcuminoids such as curcumin, demethoxycurcumin and bisdemethoxycurcumin¹⁷ are the new molecules currently under investigation.

Novel drug targets under investigation

Some of the newly investigated antileishmanial drug targets are trypanothione reductase/peroxidase, inositol phosphorylceramide synthase, fumarate reductase, microtubule associated protein (MAP2), squalene synthase, cysteine proteases, methionine aminopeptidase 2 (MetAP-2), protein kinases, signal peptidase type I (SPase I)¹⁸.

Tools for identification of drug targets

The main contributions of the last decades of the past

century are associated with the tremendous rationalization of the drug discovery process with the help of modern technologies. Previous drug discovery processes depended largely on the phenotypic observations of the effects of a natural or a synthetic product on pathological conditions. This was followed by biological testing by *in vivo* experiments. During the 21st century the exploration of the Leishmanial genome has changed the whole concept of drug discovery. Genome based approach led to thousands of new targets. This genomic methodology coupled with the combinatorial chemistry and High Throughput Screening (HTS) would yield hundreds of drug targets. The drug discovery process involves target identification, target validation, target based identification of new chemical entities (NCE's), lead optimization and clinical development (Fig. 4).

Currently, the discovery of compounds to combat tropical diseases is based on three strategies: label extension, piggy-back discovery and *de novo* drug discovery.

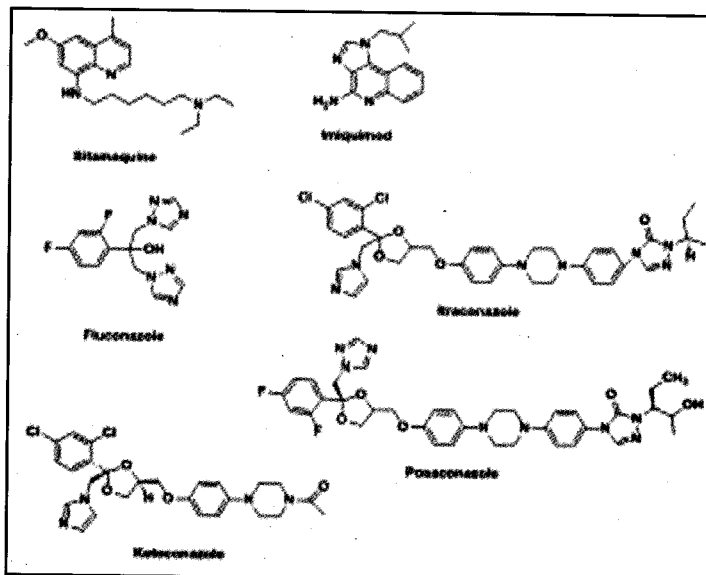


Fig. 2. Structure of drugs in development and clinical trials

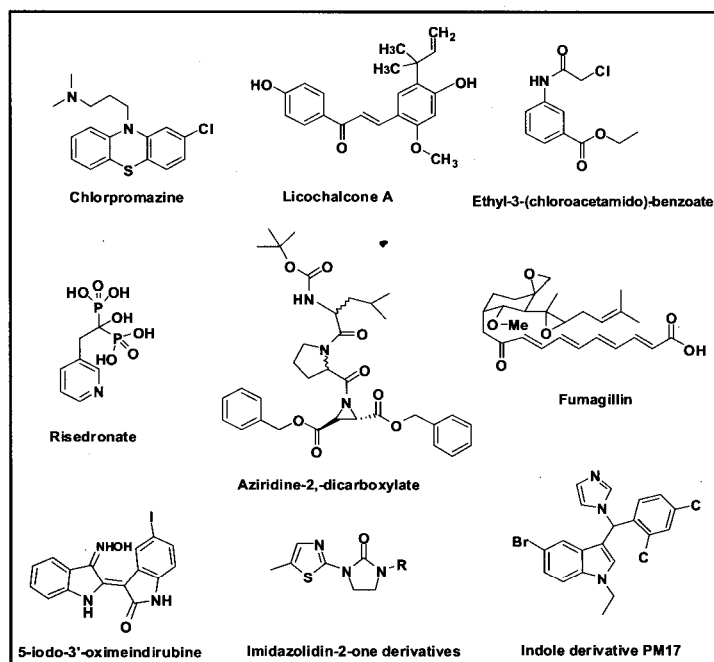


Fig. 3. Chemical structures of new lead compounds.

New indications for existing drugs: Label Extension

Given the economics of drug development for tropical diseases, label extension approach has been very attractive for fast-tracking drug discovery. These "label extension" studies typically follow a double blind randomized placebo controlled trial of a new drug, or extending the indication of existing treatments for other human and animal illness to tropical diseases¹⁹. This approach has resulted in some important anti-parasitic drugs in use today, such as ivermectin for filariasis/onchocerciasis, praziquantel for schistosomiasis, and antibiotics for malaria^{20, 21}. The azole which were developed as antifungal drugs, have undergone several trials for CL and VL with conflicting results, depending on the species. Although label extension studies provide rigorous information on safety and tolerability of potential new drugs, unexpected toxicities may arise in patients suffering from a tropical disease.

Piggy back

The 'piggy-back' strategy uses a molecular target from the parasite that has been already explored in other organisms; a combinatorial library of compounds may be investigated to identify the best anti-parasitic lead. This approach had identified promising drugs such as miltefosine for leishmaniasis and eflornithine for African trypanosomiasis, both originally developed as anti-cancer drugs¹⁹.

De novo drug discovery

De novo drug discovery involves target selection and target identification by genomics/proteomics data, target based validation of new chemical entities (NCE's), followed by drug development.

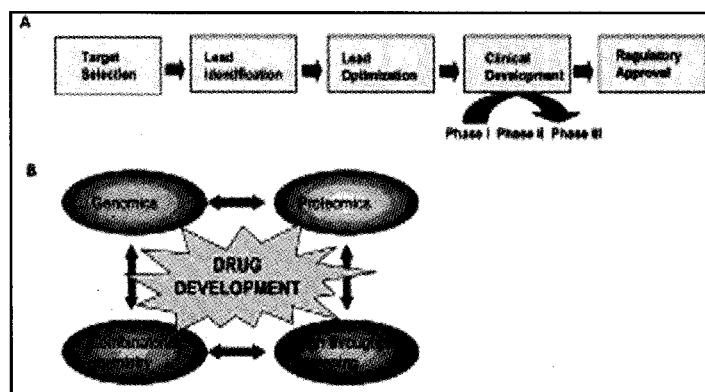


Fig. 4. A. Classical pathway of drug discovery and development process; B. The four newly emerged areas strengthen the drug development process in the 21st century.

Drug target selection

A good drug target must be expressed at the relevant stage in the parasite life cycle and ideally fulfil one or more of the following criteria: be unique to the parasite, be essential for the parasite survival or be essential for the parasite virulence. It is possible to exploit parasite biochemical pathways that are shared with humans taking advantage of structural differences between the human and parasite enzymes, in addition to pathways and enzymes unique to the parasite. For example sterol biosynthesis in trypanosomatidae is an attractive target because it differs from the mammalian host in that final product is ergosterol rather than cholesterol.

Drug target identification and validation

The different tools for identification of novel targets are: genomics, proteomics and microarray.

Genomics

The completion of genome sequencing of three kinetoplastid species (*L. major*, *T. cruzi* and *T. brucei*) provides a plethora of new targets for drug discovery^{22, 23}. Comparative genomic studies allow the identification of molecules or biochemical pathways that have already been targeted successfully in other pathogens²⁴. Besides the potential role to reveal essential pathways to be blocked, the comparative analysis of these genomes may indicate exclusive non-essential enzymes that could be used to convert a pro-drug in an active compound inside the parasite. Purine salvage, peroxisome biogenesis, glycolysis and trypanothione redox-system are metabolic pathways that diverge between parasite and host. Therefore, several components of these pathways have been investigated and are target candidates to be tested in rational anti-leishmania drug discovery platforms. The massive increase of genomic data for pathogens that cause tropical diseases has created new opportunities for drug discovery. However, the data must be effectively integrated and available in a

Table 2. Websites of interest: genome data of parasites

Leishmania spp	Trypanosoma spp	WHO/TDR list of potential drug targets
<p>Leishmania Genome Database http://www.genedb.org/genedb/leish/index.jsp</p> <p>Leishmania Genome Project http://www.ebi.ac.uk/parasites/leish.html</p> <p>Leishmania at Sanger Institute http://www.sanger.ac.uk/Projects/L_major/</p>	<p><i>T. brucei</i> Genome Project http://parsun1.path.cam.ac.uk</p> <p><i>T. brucei</i> at Sanger Institute http://www.sanger.ac.uk/Projects/T_brucei/GSS_ESTs/ribohits.shtml</p> <p><i>T. brucei</i> at TIGR http://www.tigr.org/tdb/mdb/tbdb/index.shtml</p> <p><i>T. brucei</i> Gene Database http://www.genedb.org/genedb/try/index.jsp</p> <p>The Kinetoplastid Proteome http://www.ebi.ac.uk/parasites/KinetoGN/Proteome/proteome.html</p> <p><i>T. cruzi</i> Genome Project http://www.dbbm.fiocruz.br/genome/tcruzi/tcruzi.html</p> <p><i>T. cruzi</i> Genome Project http://www.tigr.org/tdb/mdb/tcdb/</p>	<p><i>L. major</i> http://www.who.int/tdr/research/progress/leish_str/drugs.htm</p> <p><i>P. falciparum</i> http://www.who.int/tdr/research/progress/mal_str/drugs.htm</p> <p><i>T. brucei</i> http://www.who.int/tdr/research/progress/try_str/drugs.htm</p>

friendly-user mode. The tropical disease research TDR Targets database (<http://tdrtargets.org>) was created to facilitate the integration of data emerging from such studies and to help identify candidate drug targets. Some of the websites of interest are listed in Table 2. The most comprehensive yield of drug targets results from applying both loss of function genetics (LOF) and gain of function (GOF) strategies, as these complementary approaches often identify different target sets.

Loss-of-function genetics (LOF)

The emerging LOF techniques fall into three major classes on the basis of the mechanism of gene inactivation at the level of, first, the gene, second, the transcript, third, the protein. Targeted gene deletion approach is one of the methods to study LOF genetics of the organism. The proteins explored as drug target by this method are Glyoxalase I²⁵, deoxyhypusine synthase²⁶, δ -glutamylcysteine synthetase²⁷, spermidine synthetase²⁸, ornithine decarboxylase²⁹, N-myristoyltransferase³⁰, glucose transporters³¹ etc.

LOF, by decreasing RNA transcript level, provides an alternative approach to reduce gene expression in the cell. Three distinct RNA-targeting approaches – ribozymes, antisense (AS) oligonucleotides, and small interfering RNA (siRNA) – have been developed. It was reported that not only HIV, but also the replication of SARS-CoV, Chronic Hepatitis B and C, measles virus, Respiratory syncytial virus (RSV), and parainfluenza virus (PIV) could be regulated by using siRNA technology³². Not only the viral diseases are the major targets for the siRNA mediated therapeutic

approach, but the pathogenic protozoans are also the target for siRNA-mediated gene therapy. It is thought that *Trypanosoma cruzi*, the protozoan that causes Chagas' disease, modulates the extracellular matrix network to facilitate infection of human cells. Silencing of laminin δ by RNAi causes a dramatic reduction in cellular infection by *T. cruzi*³³. Although siRNA silencing has got some success in some diseases, consistent *in vivo* siRNA therapy for pathogenic diseases is still in its infancy.

The third approach is to decrease target activity by LOF at the protein level. In this approach, libraries of overexpressed random peptides are used to interfere with protein function and create cellular phenotypes. One such leishmanicidal peptide is Histatin 5 (Hst5) which inhibits F_1F_0 -ATPase causing bioenergetic collapse of the parasite³⁴.

Gain-of-function genetics (GOF)

The ability to perturb cells by gene overexpression provides an important complement to the LOF approaches described above. GOF approaches can be used to identify genes capable of conferring a disease phenotype on a cell type of interest.

Proteomics

Proteomics is a valuable tool to extend our understanding of array of events of Leishmania infection and to accelerate the search for novel potential clinical associated phenotypic markers. The production of high quality proteome maps of Leishmania parasites, in combination with MS and bioinformatics tools have enabled the identification of some novel drug targets, virulence factors, and vaccine antigens for use in disease control as well as in the understanding of

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Table 3. Proteomic analysis of different *Leishmania* spp. using various proteomic tools for the identification of new targets for diagnostics, vaccine and therapeutics³⁵.

Species	Parasite stage	Proteomic tools	Inference/remarks
Ld	Prom and amast WCP	2-DE of [³⁵ S] methionine	Identified the proteins regulated during labeled <i>Leishmania</i> stage differentiation
Ld	Prom-WCP	1-D+2-D+ immunoblotting	Identification of HSP, gp63, EIF 4A, MALDI-TOF EF-2 and grp78 antigens for diagnostic purpose
Ld	Prom-SP	2-DE + MALDI-TOF	Identification of novel immunostimulatory proteins viz. elongation factor-2, p45, HSP-70, HSP-83, aldolase, enolase, triose-phosphate isomerase, protein disulfideisomerase and clareticulin to be developed as VC
Ld	Prom-WCP	2-DE + MALDI-TOF	HSP-83 and calpain- related proteins shown to be implicated in the drug-induced PCD phenotype drug resistant isolate. Possibility of developing these proteins as DT.
Li	Prom and Amast-SP	2-DE + LC-MS/MS	Isocitrate dehydrogenase and Triose phosphate isomerase highly expressed in amastigotes may be developed as DT/ VC Li Prom-WCP 2-DE + MALDI-TOF Identified new antigenic functional proteins which may be suitable targets for both vaccination and chemotherapeutic strategies such as propionil carboxilasa, ATPase beta subunit, transketolase, proteasome subunit, succinyldiamin-ophimelate succinylase, a probable tubulin alpha chain, HSP-70.
Lm	Prom-WCP	2-DE + MALDI-TOF	Possible involvement of trypanothione reductase in drug resistance mechanism as well as its identification as drug target

Ld, *L. donovani*; Li, *L. infantum*; Lm, *L. major*; WCP, whole cell protein; VC, vaccine candidate; DT, drug target, prom, prom astfote; Amast., Amastigote.

drug resistance in *Leishmania* parasites. Recent advances in the use of multidimensional LC based separations have further dramatically improved the scope and depth of proteomic analysis and have been applied to mapping out proteomes of critical pathogenic organisms such as *Leishmania*, *Mycobacterium*, and *Plasmodium* spp. Direct LC separation of tryptic peptides coupled to MS/MS (LC-MS/MS) and isotope-coded affinity tagging (ICAT) technologies are used for stage specific proteomic analysis of various *Leishmania* species (Table 3).

Toxicogenomics

Toxicogenomics deals with global changes in gene expression in response to either a drug or a toxin, and is usually measured using microarrays (mRNA transcript) (Fig.5). It has the potential to improve our understanding of pathogenicity, mechanism of drug resistance and virulence factors by identifying up/down regulated gene and characterizing the respective gene expression. Glucose transporter, GTP-binding protein, Leishmanolysin precursor, Protein Kinase, p-Glycoprotein-like protein, Proliferative cell nuclear antigen (PCNA), Glutamate dehydrogenase, Proteasome alpha 1 subunit, ABC1 transporter, Guanine nucleotide binding protein, Glucose-6-phosphate dehydrogenase, Adenosylhomocysteinase, Translation elongation factor 1-beta genes were identified through

microarray study in VL case which was reported as a promising DT. Among metabolic gene family Fructose-1,6-bisphosphate aldolase, Enolase, Protein disulfide isomerase (PDI) were proposed as intoxicating DT & VC³⁶.

Target based identification of new chemical entities (NCE's)

Combinatorial chemistry: Delivers Myriad Ligands for a Given Target

Combinatorial chemistry is a method for creating vast numbers of molecular substances, then testing them rapidly for desirable properties. The first step in combinatorial chemistry is to identify the template which is often an active compound but discarded or in limited use due to resistance, problems in solubility, tissue distribution, toxicity and so on. The chemical groups on such a template are identified based on the propensity of their reactivities and are subsequently substituted with many different functional groups for each of them. This procedure leads to a large collection of chemical substances known as the combinatorial library. Next, the combinatorial library is rapidly evaluated to find a desirable property using High-Throughput Screenings.

One approach is a combinatorial technology that involves Darwinian-type *in vitro* evolution process, which has been termed SELEX (Systematic Evolution of Ligands by Exponential Enrichment). The procedure is a highly efficient

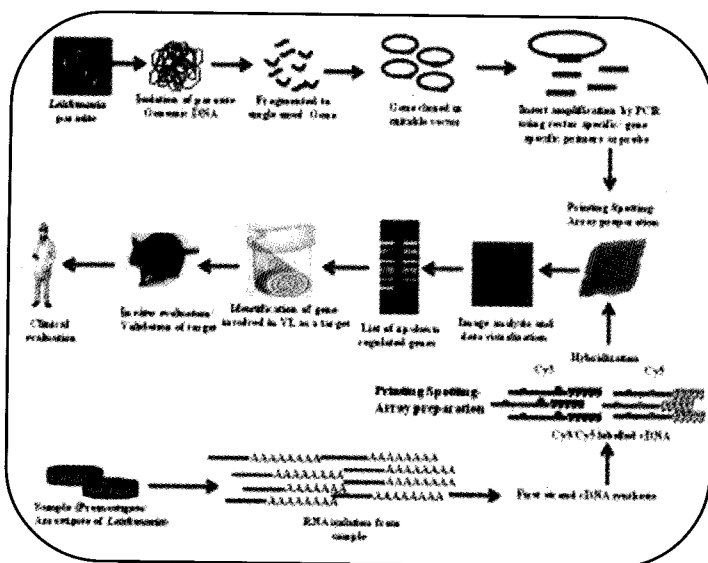


Fig. 5. Workflow of microarray for identification and development of potential drug target and vaccine candidate for VL³⁶.

method of identifying rare ligands from combinatorial nucleic acid libraries of very high complexity. It allows the selection of nucleic acid molecules with desired functions, and it has been instrumental in the identification of a number of synthetic DNA and RNA molecules, so-called "Aptamers" that recognize ligands of different chemical origin. Aptamers typically bind their target with high affinity, high specificity and have successfully been converted into pharmaceutically active compounds. Various recent examples illustrate the potential of Aptamers in infectious disease processes which work in a concentration dependent manner³².

Virtual screening (VS)

Once a molecular target has been defined, compounds that can modulate the activity of this target must be identified. The three-dimensional structure of a protein can provide a chemist with the necessary data to synthesize compounds that exhibit better potency and selectivity for a given target. A combination of chemoinformatic *in silico* screening with functional genomics, gene knockout/knockdown approaches, three-dimensional protein structure determination and their co-crystallization with small molecules are being applied for anti-bacterial and anti-parasitic drug discovery^{37, 38}. For example, the anti-influenza drug zanamivir was a result of modeling based on the crystal structure of neuraminidase³⁹. Similarly, computational docking studies were instrumental in the identification of 40 parasite-specific inhibitors of *L. mexicana* glyceraldehyde-3-phosphate dehydrogenase (GAPDH)⁴⁰. The cysteine protease inhibitor K777 is a successful example in an advanced stage of development to be used against Chagas disease⁴¹. The foregoing principles form the basis of the Structural Genomics of Pathogenic Protozoa (SGPP) consortium (<http://depts.washington.edu/sbpp/>), which aims to apply high-throughput methods to

express large numbers of proteins, and to determine three-dimensional crystal structures of proteins from *Leishmania* and other parasites. These structures are available to all researchers and will probably provide targets for structure-based lead discovery in the future.

High Throughput screening (HTS)

HTS is often the first method of choice for the interrogation of a new target molecule and has the distinct advantage that no structural information concerning the drug target is required. It is now possible to screen Library of Pharmacologically Active compounds (LOPAC) usually in a 384-well plate format. Screening of combinatorial libraries was employed to search for inhibitors of CPB2.8 "CTE protease" since cysteine proteases have been considered an attractive drug target. Several positive hits were obtained from screening of peptide isosters library and a library of peptidotriazoles. Additional antileishmanial activities were also found from screens of diamine derivatives, combinatorial library of potential inhibitors of oxidosqualene cyclase or a library screened against CRK3 cyclin-dependent kinase¹³. GDP-mannose pyrophosphorylase (GDP-MP) is an ideal *Leishmania* drug target. Screening of a library containing approximately 80,000 lead-like compounds for GDP-MP inhibitors led to the identification of potent antileishmanial compound. Another integrated approach HILCES which stands for high throughput, low-stringency, computationally enhanced small molecule screening was developed. This HILCES strategy enhanced the ability to identify novel leishmanicidal chemotypes, and, as a result, enabled to test these new chemotypes for *in vivo* leishmanicidal activity, thus effectively expanding the pool of chemical structures that could be refined as potential leishmanicidal therapies. For example, protein targets involved with cell proliferation, differentiation, invasion and motility, such as protein kinase D (gene id 5587), protein kinase C (gene id 5578), polo-like kinase 1 (gene id 5347), steroidogenic factor 1 (gene id 2516) and phosphatase regenerating liver-1 (gene id 7803) were found by this strategy⁴². Another similar filtering approach known as rapid elimination of swill (REOS) has been designed to remove from a library those compounds that have unfavorable characteristics relating to absorption, distribution, metabolism, excretion and other properties associated with poor selectivity, promiscuous binding or poor uptake by cells. Computational models are widely available for predicting ADME/Tox properties using software for either custom-model building or pre-built modeling studies [Cerius²™ ADME (<http://www.accelrys.com>) and KnowItALL™ (<http://www.bio-rad.com>)].

Development of a drug candidate

A major decision gate on the road to drug development is whether there are any properties of the drug candidate that would make its development difficult. Issues of bioavailability,

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metabolic processing, toxicity and interactions with other drugs have to be considered. In order to increase the success rate of de novo drug discovery it is necessary to implement coordination mechanisms and a complex network of laboratories to allow going from the development of a prioritized drug target portfolio to compound screening, medicinal chemistry and pharmacokinetics.

Conclusion

Recent advances in our understanding of parasite biology and immunity have not translated to measurable clinical outcomes. There is still no antileishmanial vaccine and despite identification of a multitude of novel drug candidates none of them currently undergoing clinical evaluation. The classic treatment for leishmaniasis still relies on pentavalent antimony, with a handful of a second line therapy drugs used in case of antimonial failure. One success has been the introduction of miltefosine, however, there is still a need for new drugs to treat other forms of the disease and to provide alternatives. The advent of genomic sciences, proteomics, combinatorial chemistry and high throughput screening has led to the modern concept of drug discovery. All these highly efficient techniques explore thousands of drug target molecules. These initiatives available are likely to lead to a dramatic change in translational research and there are reasons to be optimistic regarding the chances of success and cost-effectiveness of the process leading to novel drugs against leishmaniasis.

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