Physiological consequences of drug resistance in *Leishmania* and their relevance for chemotherapy

Alicia Ponte-Sucre

Laboratory of Molecular Physiology, I.M.E., Facultad de Medicina, Universidad Central de Venezuela.

Address: PO Box 68256, Caracas 1062-A, Venezuela.

E-mail: aiponte@reacciun.ve

Abstract

In the early twentieth century, infectious diseases were a leading cause of death worldwide. Through the following years, morbidity and mortality caused by infectious diseases decreased considerably in the developed world, but not in the developing world, where infectious diseases remain an important reason for concern. For example, leishmaniosis has become into a serious Third World problem. This is mainly due to an increasing frequency of drug-resistance in *Leishmania* and an enhanced risk of co-infection with HIV. Drug-resistance is usually associated with an increased expression of specific P-glycoproteins involved in membrane transport. The present review summarizes information which shows that drug-resistance is also associated with changes in physiological events such as parasite infectivity, incorporation of metabolites, xenobiotics conjugation and traffic, intracellular metabolism, host-parasite interaction, parasite cell shape and promastigote-amastigote differentiation. Furthermore, these events may change in a coordinated manner. An understanding of these physiological events may be helpful for designing chemotherapeutic approaches to multiple cellular targets, identifying strategies to circumvent *Leishmania* drug-resistance and succesfully treating leishmaniosis.

Introduction

Infection by *Leishmania* gives rise to a variety of clinical manifestations classically labelled as visceral, cutaneous and mucocutaneous leishmaniosis, and chemotherapy is the usual therapeutic approach against this disease [1]. In order to interfere with the normal development of an intracellular parasite, a drug must reach the compartment where the parasite lives; death of the

parasite then occurs, either directly or through various cell-killing mechanisms triggered by the active drug in the host cell. The complex life cycle of *Leishmania* and the intracellular nature of some of its developmental stages make such a task more difficult. Furthermore, as some species of *Leishmania* migrate to various tissues, they can be associated with all types of leishmanial diseases [2]. The antimoniates, which are the clinical drugs most frequently employed against leishmaniosis, have been in use since the 1920's. However, antimoniates have a narrow therapeutic window due to their toxicity, and there are additional conditions which permit the persistence of *Leishmania* in the vertebrate host. In fact, the World Health Organization (WHO) [3] has pointed out that the incidence of leishmaniosis has increased since the 1980's, and that leishmaniosis has gained a relevant position worldwide among the causes of death by infectious diseases. This may be due at least partly to the following facts: a) the risk of co-infection with HIV and parasites causing visceral leishmaniosis is increasing at a high rate [3], b) sub-optimal doses of the drugs employed may induce drug-resistance, and c) lack of response of *Leishmania* to various drugs occurs with high and increasing frequency [4][5][6].

The emergence of drug-resistant *Leishmania* and the increasing spread of naturally drug-resistant species stress the importance of identifying the mechanisms directly involved in drug-resistance, as well as the physiological changes that may occur in *Leishmania* as a consequence of, or concomitantly with, the development of drug-resistance. Such physiological changes, whether or not related to drug-resistance mechanisms, contribute to the overall characteristics of the drug-resistant phenotype, and new chemotherapeutic strategies against leishmaniosis could be devised if a comprehensive understanding of drug-resistance were obtained. The significance and relevance of these physiological changes are the main subject of the present review. For the sake of completeness, the genes associated with drug-resistance in *Leishmania* are also reviewed.

The life cycle of Leishmania

All *Leishmania* species are morphologically similar and display two main developmental stages through their life cycle: the amastigote, that resides inside the reticuloendothelial cells of the vertebrate host, and the promastigote, that replicates in the gut of a phlebotomine sandfly [3][7]. The life cycle can be considered to begin when the vertebrate host is bitten by an infected insect (see Fig. 1). High densities of infectant parasites block the cardial valve at the digestive tract of the sandfly and, as the insect swallows the blood from the host, it expels the valve's content, including the parasites. The insect's saliva contains chemical factors that potentiate the

parasite infective power and exert a chemotactic effect upon reticuloendothelial cells, which are attracted to the place of innoculation. The parasites are then phagocyted by reticuloendothelial cells, and this fosters their reproduction and survival. For dermotropic *Leishmania* species the lesion remains in the skin, but for viscerotropic *Leishmania* the parasite spreads from the initial skin lesion into liver, spleen and bone marrow. The parasites profusely replicate inside the reticuloendothelial cells and eventually burst free from the infected macrophages, spreading the disease within the mammal host (Fig. 1). It is still not clear whether the host cells are actively involved in this event, are physically disrupted by the infection, or undergo apoptosis [8] [9]. As a new insect bites an infected vertebrate host, it swallows infected macrophages, and the parasites differentiate into promastigotes, migrate into the midgut, become metacyclic (infective parasites) during the next four to seven days, and migrate to the cardial valve ready to be re-inoculated into a vertebrate host.

Physical and chemical differences among the hosts where *Leishmania* lives impose radically different stress conditions that must be circumvented by the parasite's ability to adapt to diverse environments. In fact, during the development of the promastigote stage, its transformation into the metacyclic promastigote, its interaction with the host cell and the establishment of the infection, the parasite is continually activating physiological responses which guarantee its survival. There are presently insufficient studies on how the development of drug-resistance interacts with the physiological events that trigger and regulate the parasite's differentiation through its life cycle. The amastigote is the cellular form relevant for human disease (Fig. 1), but pure amastigotes are difficult to obtain in sufficient number for research. It is possible, however to take advantage of the physiological equivalence between the so-called "axenic amastigotes" and lesion-derived amastigotes [10]. Axenic amastigotes have been successfully obtained by exposing promastigotes *in vitro* to extreme temperature and pH [11]. Their transformation into amastigote-like parasites is concomitant with cellular changes that include the expression of specific stage proteins on their surface [10][12][13].

Treatment of leishmaniosis

As already mentioned, chemotherapy constitutes the main tool for the control of leishmaniosis, but it is usually slow, expensive and toxic. The available antileishmanial drugs, such as sodium stibogluconate, contain pentavalent antimony, act upon the amastigote and, in order to be

effective, should be administered over 20 or more days either intravenously, for both visceral and cutaneous leishmaniosis, or intralesionally in cutaneous leishmaniosis [3]. The antimoniates were empirically developed more than 80 years ago and their mechanism of action has remained elusive. When these drugs are ineffective, or cannot be prescribed, treatment with amphotericin B, pentamidine or paramomycin is indicated. In general, all present forms of treatment show unsatisfactory features, hence the importance of developing inexpensive, effective and rapid therapies both for visceral and for cutaneous leishmaniosis. For example miltefosine, an inhibitor of sterol biosynthesis has been recently approved as the first oral drug for visceral leishmaniosis in India. It yields cure rates of about 98 %, has negligible side effects and is used successfully to treat cases resistant to antimoniates [14]. Unfortunately, *Leishmania* can develop resistance to miltefosine, at least *in vitro* [15].

Drug-resistant cell lines, selection methods, implications

In general, resistance is defined as a decline in the efficacy of a drug against a population of parasites previously susceptible to that compound. This definition assumes that the original susceptibility of the population is known, which is not always the case for *Leishmania*. Indeed, resistance of *Leishmania* against a given drug may be either natural, or may be acquired when the parasites are exposed to sub-optimal drug doses [16]. Resistant phenotypes selected *in vitro* are usually obtained by culturing wild-type parasites under stepwise increasing drug concentrations. The wild-type parasites may be either heterogeneous (non-cloned) parasites or isolated cloned colonies with high genetic homogeneity. It is possible, particularly in non-cloned colonies, that unexpected phenotypic characteristics are expressed by the majority of parasites. Care must of course be taken not to adscribe such characteristics to a subsequently developed drug-resistance.

Genomic events associated with drug-resistance in Leishmania

As in the case of tumor cells, drug-resistance in *Leishmania* has been frequently associated with decreased cellular accumulation of the drugs. Several hypotheses for decreased drug accumulation in resistant parasites have been proposed, including one analogous to the drug efflux pump model of drug-resistant tumor cells. The increased expression of a conserved type of protein called P-glycoprotein (P-gp) has been one of the most consistent changes detected in drug-resistant tumor cells [17]. P-gp belongs to the ABC transporter family, a designation associated with the existence of a highly conserved intracellular domain that specifically binds

ATP [18]. Besides its role in drug efflux, P-gp has been implicated in the elimination of compounds from the cell plasma membrane; steroid hormone secretion, phospholipid distribution, and ATP transport [19]. Over-expression of P-gp occurs as a result of chemotherapy but, since P-gp has been found in normal cells, its physiological and pathological significance is as interesting as the question of whether and how it can be targeted to improve therapy [20].

Although multidrug-resistant (MDR) cell lines which do not express P-gp have been isolated, the expression of P-gp and the efflux of drugs against a concentration gradient usually increase in sensitive cells transfected with mouse or human P-gp cDNA [21]. Alternatively, an altered membrane partition model for decreased drug accumulation in drug-resistant cells has become popular [22]. This model suggests that decreased drug accumulation is the result of alterations in pH gradients, electrical membrane potential and perhaps other biophysical parameters, and is not necessarily a direct result of drug trafficking [22].

As will be presented in the following paragraphs, the use of gene technology has yielded findings related to gene mutation, translocation and amplification which are relevant for *Leishmania* drug-resistance. Table 1 presents a summary of the genes associated with drug-resistance in *Leishmania*. As will be discussed immediately, several of these genes are related to transport molecules in the plasma membrane.

The first MDR gene homologue discovered in *Leishmania*, *ltpgpA*, was an extra chromosomal circle (H-circle) amplified in a methotrexate-resistant *L. tarentolae* promastigote cell line [23][24]. Since then, a group of genes belonging to the P-gp gene family and whose gene products confer low levels of resistance to vinblastine, arsenite and trivalent antimoniates have been characterized in *L. tarentolae* and *L. major* [25] [26] [27]. These genes are also amplified in cell lines resistant to terbinafine and primaquine [28]. The structural conformation of their gene products is similar to that of other P-gp genes, with two hemiproteins, each with six membrane segments, 22 % homology to the human *mdr1* gene product and high levels of homology with the multidrug associated protein (MRP) [29]. Transfection of *ltpgpA* into sensitive parasites triggers an increase in the levels of polyamines and trypanothione and a decrease in the accumulation of pentostam [30][31][32][33]. The protein products of these genes play important roles as intracellular metal-thiol-transporter ATPases in the early steps of resistance [34]. In other studies, *L. tarentolae* promastigotes stepwise selected for resistance to sodium stibogluconate, and mutants resistant to antimony-containing drugs and cross-resistant to arsenite, showed amplification of a locus without significant homology to

sequences previously described [35]. Recently, in methotrexate-resistant *L. tarentolae* a deletion of folic acid transporters has also been demonstrated [36].

A gene related to P-gp in *Leishmania* is *mdr1*; its expression is frequently increased in cells with the MDR-phenotype, a result confirmed by transfection experiments in *L. enrietti* [37]. *L. tropica* resistant to daunomycin over-express, as an extra chromosomal circular location, an MDR-like gene, *ltmdr1*, thus implicated in the decreased accumulation of the drug in resistant parasites [38]. In *L. amazonensis* the longest open reading frame of the *lamdr1* gene encodes a protein consisting of two similar halves, each containing six putative transmembrane domains and one ATP-binding domain. These two halves are 91 and 78% identical, respectively, to the closely related *ldmdr1* in *L. donovani* and *lemdr1* in *L. enriettii* [39]. Transfection of *lamdr1* to wild-type parasites conferred a significant level of resistance to vinblastine, doxorubicin, and actinomycin-D. This drug specificity of *lamdr1* overlaps with, but is distinct from, that of *ldmdr1*, thus suggesting a functional diversity of *mdr1*-derived proteins among different *Leishmania* species.

In *L. donovani*, selection and isolation under the pressure of tubericidin led to the characterization of a resistant cell line which showed impaired drug transport through the plasma membrane [40]. Since tubericidin enters the cell through nucleoside transporters, point mutations in strategic transporter sites were suggested [41]. Other studies have also shown that vinblastine-resistant *L. donovani* displays cross-resistance to puromycin and anthracyclines and shows amplification of DNA fragments homologous to the mammalian MDR [42]. It has also been found that methotrexate-resistant *L. donovani* is deficient in folate-methotrexate transporters [43].

Pentamidine drug-resistance has been shown to be linked to a decreased accumulation of this compound, accompanied by a decreased transport of structurally unrelated molecules such as pyrimidine nucleotide as well as by alterations of the polyamine levels [44]. A DNA fragment which hybridized to *ltpgpA* and showed size polymorphism between the sensitive and the resistant strain was amplified [46] in *L. mexicana* made resistant to glibenclamide -a general blocker of ABC transporters including P-gp from different drug-resistant cell lines [45]. This amplification correlates with increased expression of a 185 kDa protein band in the resistant strain, faintly expressed in the sensitive strain [47], that was recognized by the antibody (F4) against the N-terminal of P-gp.

The availability of gene transfection technology in *Leishmania* originated from studies related to the amplification of the dihydrofolate reductase gene (*dhfr-ts*) in methotrexate-resistant

parasites as well as to the design and creation of shuttle vectors for stable transfection and expression of foreign DNA in these parasites [48][49]. The use of shuttle vectors has yielded findings that are relevant for *Leishmania* drug-resistance. These findings may be related to drug transport phenomena, as presented in the previous paragraphs, or may be unrelated to drug transport but nevertheless relevant for *Leishmania* drug-resistance, as will be presented forthwith. For example, *L. mexicana* made resistant to tunicamycin, a drug that selectively inhibits the enzyme N-acetylglucosamine-1-phosphate transferase (NAGT), has been shown to express higher copy numbers of an extracellular 63 kilobase (Kb) circular amplicon [50]. By means of DNA fragment transfection, a 15 Kb clone was described to confer tunicamycin-resistance to wild-type *L. amazonensis* [51]. The minimal 4.6 Kb region necessary to confer tunicamycin resistance was shown to code for NAGT, and the different transfectants overproduced a NAGT transcript of identical size [51]. As a consequence of methotrexate resistance, *L. tarentolae* preferentially shows amplification of the H locus (*ltpgpA*) and the pterin reductase gene (*ptr1*); in *L. tarentolae* made mutant for the H locus there is amplification of the dihydrofolate reductase-thymidylate synthase, or R locus [52]. Furthermore, transfection experiments using shuttle vectors with *ptr1* at the R locus and *dhfr-ts* at the H locus have shown that *ptr1* gave higher levels of methotrexate resistance than *dhfr-ts*, and that *ptr1* was invariably amplified –when present-while *dhfr-ts* was only amplified when *ptr1* was inactivated [52]. These experiments demonstrated that both the locus and the gene determine the frequency of gene amplification in *Leishmania*. In *L. donovani* resistance to mycophenolic acid, a potent inhibitor of the inosine monophosphate dehydrogenase (IMPDH), is associated with the amplification of a stable linear extrachromosomal DNA (280 Kb in size) that codes for the IMPDH gene [53]. This extrachromosomal element was subjected to large-sized deletions of its central region and the resulting size-reduced DNA elements were cloned into vectors. These vectors were transfected into mycophenolic acid-resistant *Leishmania* cells. The mitotic stability of the mutated DNA elements was then analysed over 140 days in drug-free cultures. The largest deletions (DNA fragments of 120 and 200 Kb) altered the mitotic stability and led to a progressive loss of the size-reduced DNA elements. A 30 Kb region common to both deletions was considered as involved in mitotic stability and associated to stable drug-resistance in *Leishmania*. However, sequence analysis did not show elements typical of centromeric DNA, thus suggesting that the mitotic stability of the DNA element was not associated to the presence of a centromere [53].

Finally, proteomics has been used to identify and analyse an overexpression of PTR1 in methotrexate-resistant *L. major* [54]. This constitutes the first proteomic analysis of drug-resistance in a parasite and also the clearest identification of a primary drug-resistance mechanism by means of this approach [54].

Physiological events associated with drug-resistance in **Leishmania**

As will be presented herein, there are cellular events that occur along with *Leishmania* drug-resistance that include changes in xenobiotics conjugation and traffic, cytoskeleton phosphorylation, membrane microviscosity and composition, metacyclogenesis and infectivity, and mitochondrial gene expression. It is still unclear whether these events are central to the expression of drug-resistance or rather constitute epiphenomena not directly related to drug-resistance. Also unclear is whether these events depend on the amastigote itself or are instead related to the expression of drug-resistance within the patient. Nevertheless, a comprehensive understanding of these physiological characteristics of drug-resistant *Leishmania* seems fundamental for the treatment and control of leishmaniosis.

Resistant species of *L. tarentolae* accumulate less arsenite than wild-type cells [55] and yet *in vitro* experiments have shown no difference in the rate of arsenite accumulation in everted plasma membrane-enriched vesicles prepared from wild-type or from arsenite-resistant cells [56]. In resistant promastigotes, however, there is an increased synthesis of the substrate for the transporters as well as of trypanothione, the thiol involved in the arsenical conjugation and extrusion [30][32][33]. This suggests that increased synthesis of the substrate rather than an increased number of membrane transporters might constitute a different and novel mechanism of drug-resistance. This also suggests that arsenite-resistance might be mediated by a multigen mechanism which involves *ltpgpA*, the enzyme gamma-glutamylcysteine synthetase (GSH) at the rate limiting step of glutathione biosynthesis [56], and a transporter which actively extrudes As (III) glutathione [57]. Such a multigen mechanism of drug-resistance has also been observed in vinblastine-resistant *Leishmania* [37] [42] [58].

In an arsenite-resistant *L. donovani*, the level of α tubulin expression is similar between wild-type and resistant promastigotes selected from non-cloned parasites. Differentiation into axenic amastigotes caused differences in the levels of tubulin expression between the two strains [59]. More interestingly, α and β tubulin phosphorylation was highly increased in the resistant strain [60]. It is well known that microtubules (and tubulin) play important roles in leishmanial

proliferation, cell shape and differentiation [61], and the aforementioned results suggest that the expression of drug-resistance could alter either the tubulin proteins themselves or the events leading to cytoskeletal changes that occur during parasite differentiation. As the two strains expressed different sensitivities to paclitaxel -a drug that promotes tubulin assembly-, these results suggest that changes in the expression of structural genes and in the signal transduction cascades might be related to drug sensitivity [60][61].

In *L. donovani* promastigotes resistant to amphotericin-B a decreased influx and an increased efflux of the drug, as well as a lower membrane microviscosity, have been demonstrated [62]. Notably, the prevalent lipids in resistant *L. donovani* cell plasma membrane were saturated fatty acids and an ergosterol precursor, cholesta-5,7,24-trien-3 β-ol, instead of the ergosterol normally found in wild-type parasites. On the other hand, in terbinafine-resistant *L. major* an increased expression of ergosterol biosynthetic intermediates was demonstrated [63]. Finally, in pentamidine-resistant *L. amazonensis* alterations in membrane fluidity, lipid content and loss of pentamidine binding sites have been described [64]. These results suggest that an altered membrane fluidity and changes in the extracellular exposure of receptor molecules may be relevant for drug sensitivity in resistant parasites [62][63][64].

Results presented by Sereno and Lemesre [65] indicate that no difference in the *in vitro* infectivity for resident mouse macrophages is observed between wild-type and pentamidine-resistant clones, but decreased *in vivo* and *in vitro* infectivity has been described in drug-resistant *L. donovani* [59], *L. guyanensis* [66] and *L. mexicana amazonensis* [47] [67]. Decreased lectin-induced parasite agglutination is associated with a decreased infectivity, at least in *L. guyanensis* [66], as well as with a decreased expression of the *L. mexicana* meta-1 protein, indicative of metacyclogenesis in *L. major* [68] (Silva, N., Camacho, N., Figarella, K and Ponte-Sucre, A., in preparation). In this context, the activity of acid phosphatase, an enzyme which has been described as a virulence marker in *Leishmania* [69], decreased significantly in resistant *L. mexicana* [47]. Also, there are alterations in lipophosphoglycan expression in pentamidine-resistant parasites [64] as well as an altered pattern of serine residue phosphorylation in *L. mexicana* resistant to ABC transporter blockers [47]. Altogether these results suggest that the development of drug-resistance in *Leishmania* might involve alterations in its differentiation into metacyclic infective parasites.

In pentamidine-resistant parasites a modification of mitochondrial membrane potential has been demonstrated [64]. Also, a point mutation in the mitochondrial apocytochrome b (Cyb) gene has been

associated to antimycin-A resistance in *L. tarentolae* [70]. However, in *L. mexicana amazonensis* there were no significant differences in respiration rate and cellular ATP content between arsenite-resistant and wild-type cells [71]. These results suggest that the mitochondrial genome might mutate as a consequence of drug pressure but respiration and ATP seem not to be prime events associated with drug-resistance.

Conclusion

The emergence of drug-resistance represents one of the most serious problems in the control of infectious diseases. Treatment failure in kala-azar, mucosal leishmaniosis and some forms of cutaneous leishmaniosis, occurring in 50-70 % of the patients [72], is becoming a common problem in many endemic areas. In some cases, this could be attributed to altered drug pharmacokinetics, reinfection, or immunologic compromise of the host. However, there are now strong indicators which suggest that it may be partly due to drug-resistance [72]. Results summarized in the present review show that the overexpression of membrane-bound ATP binding cassette proteins is linked to drug-resistance in *Leishmania*. These transport systems modulate the efflux or intracellular trafficking of chemotherapeutic agents. Furthermore, the development of drug-resistance includes alteration of other biochemical and physiological parameters. As summarized in Fig. 2, mechanisms described so far involve fundamental physiological functions such as drug transport (MDR and MRP), parasite's infectivity (lipophosphoglycan, acid phosphatase and meta-1 expression), incorporation of metabolites considered to be fundamental for the parasite survival (folates and nucleosides), xenobiotics conjugation and traffic (trypanothione and Cyb expression), intracellular metabolism (dihydrofolate reductase-thymidylate synthetase, N-acetylglucosamine-1-transferase and pterin transferase), host-parasite interaction (membrane fluidity and lectin agglutination) and parasite cell shape and promastigote-amastigote differentiation (tubulin phosphorylation). More interestingly, mechanisms other than the frequent amplification of transport systems mediated by MDR or MRP are preferentially linked; this is the case of the pterin transferase gene and the dihydrofolate reductase-thymidylate synthetase complex in methotrexate-resistant parasites, or the gamma-glutamylcysteine synthetase enzyme and a transporter which actively extrudes As (III) glutathione in arsenite-resistant parasites.

These data highlight the large effort that has been invested in understanding the changes associated with drug-resistance in *Leishmania*, but questions still unanswered are whether these physiological

events a) are central to the expression of drug-resistance, b) constitute epiphenomena not directly related to drug-resistance, c) are expresed by cloned promastigotes before being selected, d) are expressed by the amastigote and therefore affect the intracellular survival of the parasite, and e) are related to the expression of drug-resistance within the patient. Nevertheless, it is hoped that knowledge of such variety of physiological mechanisms will be translated into a more effective treatment and control of leishmaniosis.

Acknowledgements

References

[1] Barret MP, Mottram JC, Coombs, GH. Recent advances in identifying and validating drug targets in trypanosomes and leishmanias. *Trends Microbiol* 1999, 7: 82-88

[2] Barral A, Pedral-Sampaio D, Grimaldi Jr G, Momen H, McMahon-Pratt D, Ribeiro de Jesus A, Almeida R, Badaro R, Barral-Neto M, Carvalho EM. Leishmaniasis in Bahia, Brazil: evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease. *Amer J Trop Med Hyg* 1991, 44: 536- 546

[3] http://www.who.int/tdr/diseases/leish/default.htm

[4] Borst P. Genetic mechanisms of drug resistance. A review. *Acta Oncol* 1991, 30: 87-105

[5] Borst P, Ouellette M. New mechanisms of drug resistance in parasitic protozoa. *Ann Rev Microbiol* 1995, 49: 427-460

[6] Ullman B. Multidrug resistance and P-glycoproteins in parasitic protozoa. *J Bioenerg Biomembr* 1995, 27: 77- 84

[7] Killick-Kendrick R, Molineux W. Transmission of leishmaniasis by the bite of a phlebotomine, possible mechanisms. *Trans R Soc Trop Med Hyg* 1981, 75: 152-154

[8] Chang KP, Reed SG, McGwire BS, Soong L. *Leishmania* model for microbial virulence: the relevance of parasite multiplication and pathoantigenicity. *Acta Trop* 2003, 85: 375-390.

[9] DosReis G. Susceptible Hosts: a resort for parasites right in the eye of the immune response. *An Acad Bras Cienc* 2000, 72: 79-82

Kinetoplastid Biology and Disease 2003, 2:14 (http://www.kinetoplastids.com/home/).

[10] Ismaeel AY, Garmson JC, Molyneux DH, Bates PA. Transformation, development, and transmission of axenically cultured amastigotes of *Leishmania mexicana in vitro* and in *Lutzomyia longipalpis Am J Trop Med Hyg* 1998, 59: 421-425

[11] Zilberstein D, Shapira M. The role of pH and temperature in the development of *Leishmania* parasites. *Ann Rev Microbiol* 1994, 48: 449-470

[12] Marchesini N, Docampo R. A plasma membrane P-type H atpase regulates intracellular pH in *Leishmania mexicana amazonensis. Mol Biochem Parasitol* 2002, 119: 225-236

[13] Turco SJ, Sacks DL. Expression of a stage-specific lipophosphoglycan in *Leishmania major* amastigotes. *Mol Biochem Parasitol* 1991, 45: 91- 99

[14] Jha TK, Sundar S, Thakur CP, Bachmann P, Karbwang J, Fischer C, Voss A, Berman J. Miltefosine, an oral agent for the treatment of indian visceral leishmaniasis. *N Eng J Med* 1999, 341: 1795-1800

[15] Perez-Victoria JM, Perez-Victoria FJ, Parodi-Talice A, Jimenez IA, Ravelo AG, Castanys S, Gamarro F. Alkyl-lysophospholipid resistance in multidrug-resistant *Leishmania tropica* and chemosensitization by a novel P-glycoprotein-like transporter modulator. *Antimicrob Agents Chemother* 2001, 45: 2468-2474

[16] Cohen ML. Epidemiology of drug resistance: Implications for a post-antimicrobial era. *Science* 1992, 257: 1050-1082

[17] Higgins CF. ABC transporters: from microorganisms to man. *Ann Rev Cell Biol* 1992, 8: 67-113

[18] Doige CA, Ames GF. ATP-dependent transport systems in bacteria and humans: relevance to cystic fibrosis and multidrug resistance. *Ann Rev Microbiol* 1993, 47: 291-319

[19] Orlowski S, Garrigos, M. Multiple recognition of various amphiphilic molecules by the multidrug resistance P-glycoprotein: molecular mechanisms and pharmacological consequences coming from functional interactions between various drugs. *Anticanc Res* 1999, 19: 3109-3123

[20] Bradshaw DM, Arceci RJ. Clinical relevance of transmembrane drug efflux as a mechanism of multidrug resistance. *J Clin Oncol* 1998, 16: 3674-3690

[21] Endicott JA, Ling V. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Ann Rev Biochem* 1989, 58: 137-171

[22] Roepe PD, Martiney JA. Are ion exchange processes central to understanding drug-resistance phenomena? *Trends Pharmacol Sc* 1999, 20: 62-65

[23] Ouellette M, Fase-Fowler F, Borst P. The amplified H circle of methotrexate-resistant *Leishmania tarentolae* contains a novel P-glycoprotein gene. *The EMBO J* 1990, 9: 1027-1033

[24] Ouellette M, Hettema E, Wust D, Fase-Fowler F, Borst P. Direct and inverted DNA repeats associated with P-glycoprotein gene amplification in drug resistant *Leishmania. The EMBO J* 1991, 10: 1009-1016

[25] Ouellette M, Borst P. Drug resistance and P-glycoprotein gene amplification in the protozoan parasite *Leishmania. Res Microbiol* 1991, 142: 737-746

[26] Légaré D, Hettema E, Ouellette M. The P-glycoprotein-related gene family in *Leishmania. Mol Biochem Parasitol* 1994, 68: 81-91

[27] Papadopoulou, B., Roy, G., Dey, S., Rosen, B.P. & Ouellette, M. Contribution of the *Leishmania* P-glycoprotein-related gene ltpgpA to oxyanion resistance. *J Biol Chem* 1994, 269: 11980-11986

[28] Ellenberger TE, Beverley SM. Multiple drug resistance and conservative amplification of the H region in *Leishmania major*. *J Biol Chem* 1989, 264: 15094-150103

[29] Cole SP, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG. Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* 1994, 54: 5902-5910

[30] Mukhopadhyay R, Dey S, Xu N, Gage D, Lightbody J, Ouellette M, Rosen BP Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. *Proc Natl Acad Sci* 1996, 93: 10383-10387

[31] Papadopoulou B, Roy G, Ouellette M. A novel antifolate resistance gene on the amplified H circle of *Leishmania. The EMBO J* 1992, 11: 3601- 3608

[32] Haimeur A, Guimond C, Pilote S, Mukhopadhyay R, Rosen BP, Poulin R, Ouellette M. Elevated levels of polyamines and trypanothione resulting from overexpression of the ornithine decarboxylase gene in arsenite-resistant *Leishmania*. *Molec Microbiol* 1999, 34: 726- 735

[33] Haimeur A, Brochu C, Genest P, Papadopoulou B, Ouellette M. Amplification of the ABC transporter gene PGPA and increased trypanothione levels in potassium antimonyl tartrate (SbIII) resistant *Leishmania tarentolae*. *Mol Biochem Parasitol* 2000, 108: 131-135

[34] Légaré D, Richard D, Mukhopadhyay, R., Stierhof Y-D, Rosen B.P., Haimeur A, Papadopolou B, Ouellette M. The *Leishmania* ATP-binding casstte protein PGPA is an intracellular metal-thiol transporter ATPase. *J Biol Chem* 2001, 276: 26301-26307

[35] Haimeur A, Ouellette M. Gene amplification in *Leishmania tarentolae* selected for resistance to sodium stibogluconate. *Antimicrob Agents Chemother* 1998, 42: 1689-1694

 [36] Richard D, Kundig C, Ouellette M. A new type of high affinity folic acid transporter in the protozoan parasite *Leishmania* and deletion of its gene in methotrexate-resistant cells. *J Biol Chem* 2002, 277: 29460-29467

[37] Chow LM, Wong AK, Ullman B, Wirth DF. Cloning and functional analysis of an extrachromosomally amplified multidrug resistance-like gene in *Leishmania enriettii*. *Mol Biochem Parasitol* 1993, 60: 195-208

[38] Chiquero MJ, Perez-Victoria JM, O´Valle F, González-Ros JM, del Moral RG, Ferragut JA, Castanys S, Gamarro F. Altered drug membrane permeability in a multidrug-resistant *Leishmania tropica* line. *Biochem Pharmacol* 1998, 55: 131-139

[39] Katakura K, Iwanami M, Ohtomo H, Fujise H, Hashiguchi Y. Structural and functional analysis of the LaMDR1 multidrug resistance gene in *Leishmania amazonensis*. *Biochem Biophys Res Commun* 1999, 255: 289-294

[40] Hendrickson N, Sifri CD, Henderson DM, Allen T, Wirth DF, Ullman B. Molecular characterization of the ldmdr1 multidrug resistance gene from *Leishmania donovani*. *Mol Biochem Parasitol* 1993, 60: 53-64

[41] Vasudevan G, Ullman B, Landfear SM. Point mutations in a nucleoside transporter gene from *Leishmania donovani* confer drug resistance and alter substrate selectivity. *Proc Natl Acad Sci USA* 2001, 98: 6092-6097

[42] Henderson DM, Sifri CD, Rodgers M, Wirth DF, Hendrickson N, Ullman B. Multidrug resistance in *Leishmania donovani* is conferred by amplification of a gene homologous to the mammalian *mdr1* gene. *Mol Cell Biol* 1992, 12: 2855-2865

[43] Kaur K, Coons T, Emmett K, Ullman B. Methotrexate-resistant *Leishmania donovani* genetically deficient in the folate methotrexate transporter. *J Biol Chem* 1998, 263: 7020-7028

[44] Basselin M, Lawrence F, Robert-Gero M. Altered transport properties of pentamidine-resistant *Leishmania donovani* and *L. amazonensis. Parasitol Res* 1997, 83: 413-418

[45] Golstein PE, Boom A, van Geffel J, Jacobs P, Masereel B, Beauwens R. P-glycoprotein inhibition by glibenclamide and related compounds. *Pflugers Arch* 1999, 437: 652-660

[46] Ponte-Sucre A, Campos Y, Vazquez J, Moll H, Mendoza-León A. Sensitivity of *Leishmania spp*. to glibenclamide and 4-aminopyridine: a tool for the study of drug resistance development. *Mem Inst Oswaldo Cruz* 1997, 92: 601- 606

[47] García N, Figarella K, Mendoza-León A, Ponte-Sucre A. Changes in the infectivity, pyruvate kinase and acid phosphatase activity and p-glycoprotein expression in glibenclamide resistant *Leishmania mexicana. Parasitol Res* 2000, 86: 899-904

[48] Kapler GF, Coburn CM, Beverley SM. Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase region sufficient for extrachromosomal replication and expression. *Mol Cell Biol* 1990, 10: 1084-1094

[49] Callahan HL, Beverley SM. A member of the aldoketo reductase family confers methotrexate resistance in *Leishmania. J Biol Chem* 1992, 267: 24165-24168

[50] Detke S, Chaudhuri G, Kink JA, Chang KP. DNA amplification in tunicamycin-resistant *Leishmania mexicana:* multicopies of a single 63-kilobase supercoiled molecule and their expression. *J Biol Chem* 1988, 263: 3418-3424

[51] Liu X, Chang KP. The 63-kilobase circular amplicon of tunicamycin-resistant *Leishmania amazonensis* contains a functional N-acetylglucosamine-1-phosphate transferase gene that can be used as a dominant selectable marker in transfection. *Molec Cell Biol,* 12: 4112-4122

[52] Kündig C, Leblanc É, Papadopoulou B, Ouellette M. Role of the locus and of the resistance gene on gene amplification frequency in methotrexate resistant *Leishmania tarentolae. Nuc Acids Res* 1999, 27: 3653-365

[53] Dubessay P, Ravel C, Bastien P, Lignon MM, Ullman B, Blanieau C. Effect of large targeted deletions on the mitotic stability of an extra chromosome mediating drug resistance in *Leishmania. Nuc Acids Res* 2001, 29: 3231-3240

[54] Drummelsmith J, Brochu V, Girard I, Messier N, Ouellette M. Proteome mapping of the protozoan parasite *Leishmania* and application to the study of drug targets and resistance mechanisms. *Mol Cell Proteomics* 2003, 2:146-155

[55] Dey S, Papadopolou B, Haimeur A, Roy G, Grondin K, Dou D, Roser BP, Ouellette M. High level arsenite resistance in *Leishmania tarentolae* is mediated by an active extrussion system. *Mol Biochem Parasitol* 1994. 67: 49-57

[56] Grondin K, Haimeur A, Mukhopadhyay R, Rosen B P, Quellette M. Co-amplification of the g-glutamylcysteine synthetase gene gsh1 and of the ABC transporter gene pgpA in arsenite-resistant *Leishmania tarentolae The EMBO J* 1997, 16: 3057-3065

[57] Dey S, Ouellette M, Lightbody J, Papadopoulou B, Rosen BP. An ATP-dependent As(III)-glutathione transport system in membrane vesicles of *Leishmania tarentolae*. *Proc Natl Acad Sci USA* 1996, 93: 2192-2197

[58] Wong AK, Chow LM, Wirth DF. A homologous recombination strategy to analyze the vinblastine resistance property of the V-circle in *Leishmania*. *Mol Biochem Parasitol* 1994, 64: 75-86

[59] Prasad V, Kumar SS, Dey C. Resistance to arsenite modulates levels of α-tubulin and sensitivity to paclitaxel in *Leishmania donovani. Parasitol Res* 2000, 86: 838-842

[60] Prasad V, Dey CS. Tubulin is hyperphosphorylated on serine and tyrosine residues in arsenite-resistant *Leishmania donovani* promastigotes. *Parasitol Res* 2000, 86: 876-880

[61] Chan MM, Triemer RE, Fong D. Effect of the anti-microtubule drug oryzalin on growth and differentiation of the parasitic protozoan *Leishmania mexicana*. *Differentiation* 1991, 46: 15-21

[62] Mbongo N, Loiseau P, Billio MA, Robert-Gero M. Mechanism of amphotericin B resistance in *Leishmania donovani* promastigotes. *Antimicrob Agents and Chemother* 1998, 42: 352-357

[63] Cotrim PC, Garrity LK, Beverley SM. Isolation of genes mediating resistance to inhibitors of nucleoside and ergosterol metabolism in *Leishmania* by overexpression/selection. *J Biol Chem* 1999, 274: 37723-37730

[64] Basselin M, Robert-Gero M. Alterations in membrane fluidity, lipid metabolism, mithocondrial activity and lipophosphoglycan expression in pentamidine-resistant *Leishmania. Parasitol Res* 1998, 60: 78-83

[65] Sereno F, Lemesre FJ. *In vitro* life cycle of pentamidine-resistant amastigotes: Stability of the chemoresistant phenotypes is dependent on the level of resistance induced. *Antimicrob Agents and Chemother* 1997, 41: 1898-1903

[66] Gazola KC, Ferreira AV, Anacleto C, Michalik MS, Andrade A, Moreira ES. Cell surface carbohydrates and *in vivo* infectivity of glucantime sensitive and resistant *Leishmania (viannia) guyanensis* cell lines. *Parasitol Res* 2001, 87: 935-940

[67] Silva N, Ponte-Sucre A. ABC proteins in *Leishmania mexicana*: Modulation of parasite host cell interaction. *Arch Ven Farmacol Ter* 2001, 20: 134-138

[68] Berberich C, Marín M, Ramírez JR, Muskus C, Vélez ID. The metacyclic stage-expressed Meta-1 gene is conserved between Old and New World *Leishmania* species. *Memór Inst Oswaldo Cruz* 1998, 93**:** 819-821

[69] Vannier-Santos M, Martiny A, Meyer-Fernandez JR, de Souza W. Leishmanial protein kinase c modulates host cell infection via secreted acid phopshatase. *Eur J Cell Biol* 1995, 67: 112-119

[70] Schnaufer A, Sbicego S, Blum B. Antimycin A resistance in a mutant *Leishmania tarentolae* strain is correlated to a point mutation in the mitochondrial apocytochrome b gene. Curr Genet 2000, 37: 234-241

[71] Singh AK, Lee ST. Status of respiration and ATP content in arsenite resistant *Leishmania mexicana amazonensis. Microb Pathog* 1999, 26: 171-174

[72] Grogl M, Thomason, T.N., Franke, E.D. Drug resistance in leishmaniasis: its implications in systemic chemotherapy of cutaneous and mucocutaneous disease. *Am J Trop Med Hyg* 1991, 47: 117-126

[73] Gueiros-Filho FJ, Viola JP, Gomes FC, Farina M, Lins U, Bertho AL, Wirth DF, Lopes UG. *Leishmania amazonensis*: multidrug resistance in vinblastine-resistant promastigotes is associated with rhodamine 123 efflux, DNA amplification, and RNA overexpression of a Leishmania mdr1 gene. *Exp Parasitol* 1995, 81: 480-490

[74] Lye LF, Hsieh YH, Su KE, Lee ST. Cloning and functional analysis of the ribonucleotide reductase gene small subunit from hydroyurea-resistant *Leishmania mexicana amazonensis. Mol Biochem Parasitol* 1997, 90: 353-358

[75] Chiquero MJ, Olmo A, Navarro P, Rios-Perez LM, Castanys S, Gonzalez Pacanowska D, Gamarro F. Amplification of the H locus in *Leishmania infantum. Biochim Biophys Acta* 1994, 1227: 188-194

[76] Gamarro F, Chiquero MJ, Amador MV, Legare D, Ouellette M, Castanys S. P-glycoprotein overexpression in methotrexate-resistant *Leishmania tropica*. *Biochem Pharmacol* 1994, 47: 1939-1947
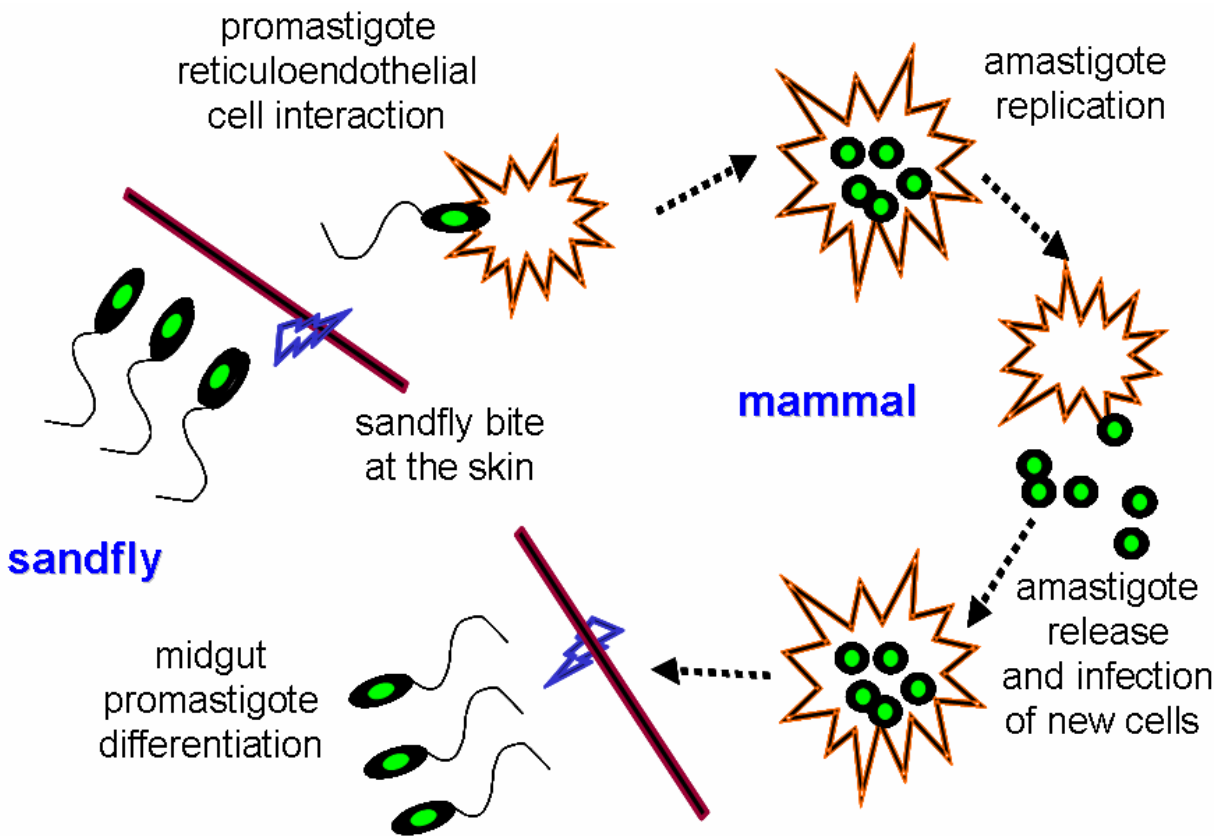
Figure 1: The *Leishmania* life cycle.

The *Leishmania* life cycle begins when the vertebrate host is bitten by the infected insect. Then the inoculated parasites are phagocyted by the reticuloendothelial cells, the intracellular parasites replicate, and eventually burst free from the infected macrophages, spreading the disease within the mammal host. As a new insect bites an infected vertebrate host, it swallows infected macrophages, the parasites are then released, differentiate into promastigotes, migrate into the midgut, become metacyclic (infective parasites) during the next four to seven days and migrate to the cardial valve ready to be re-inoculated into a vertebrate host.
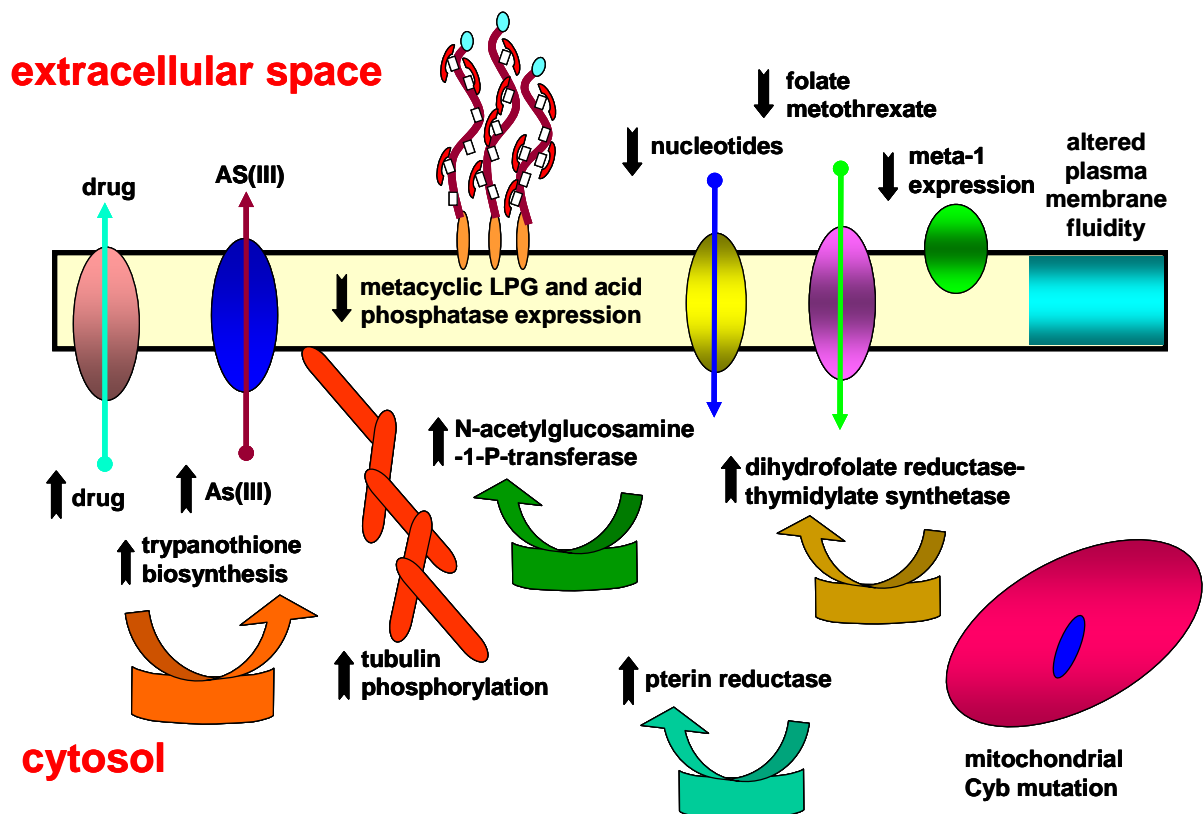
extracellular space

drug

AS(III)

folate
metothrexate

nucleotides

meta-1
expression

altered
plasma
membrane
fluidity

metacyclic LPG and acid
phosphatase expression

drug

As(III)

N-acetylglucosamine
-1-P-transferase

dihydrofolate reductase-
thymidylate synthetase

trypanothione
biosynthesis

tubulin
phosphorylation

pterin reductase

mitochondrial
Cyb mutation

cytosol

Figure 2: Physiological events associated with drug-resistance in *Leishmania*.

The physiological events associated with drug-resistance in *Leishmania* include changes in P-gp expression, parasite infectivity (lipophosphoglycan, acid phosphatase and meta-1 expression), incorporation of metabolites fundamental for the parasite survival (folates and nucleosides), xenobiotics conjugation and extrusion (trypanothione and Cyb expression), intracellular metabolism (dihydrofolate reductase-thymidylate synthetase, N-acetylglucosamine-1-transferase and pterin transferase), host-parasite interaction (membrane fluidity) and parasite cell shape and promastigote-amastigote differentiation (tubulin phosphorylation). Black arrows indicate whether a given parameter is increased or decreased in the resistant parasite.

Table 1

Genes associated to drug resistance in *Leishmania*

Species	Gene	Resistance to	Cross resistance	Reference
L. amazonensis	*lamdr*	vinblastine	adriamycin	73
		vinblastine		
		doxorubicin		39
		actinomycin-D		
	KDNA	pentamidine		64
	ribonucleotide			
	reductase	hydroxyurea		74
	gene			
	NAGT	tunicamycin		51
L. donovani	*ldNTt1.1*	tubericidin		41
	ldmdr1	vinblastine	puromycin	
			anthracyclins	40, 42
		methotrexate		43
	IMPDH		mycophenolic acid	53
L. enriettii	*lemdr*	vinblastine	puromycin	37
L. infantum	H-circle	methotrexate		75
L. major	H-circle	primaquine	methotrexate	28
		terbinafine		
	SQS1	terbinafine		
	SQS1	itraconazole		
	TOR	tubericidin		54, 63
	TUB2	tubericidin		
	DHFR-TS	antifolates		
	PTR1	antifolates		
L. tarentolae	H-circle	methotrexate		23, 24
	ltpgpA-E	vinblastine		
		arsenite		25, 26, 27, 33
		antimoniates		
	gsh	arsenite		57
	Cyb	antimycin-A		70
	DHFR-TS	folates		
	Ltdth	methotrexate		31, 36, 52
	PTR1			
L. tropica	*ltpgpE*	methotrexate		76
	ltmdr	daunomycin		38