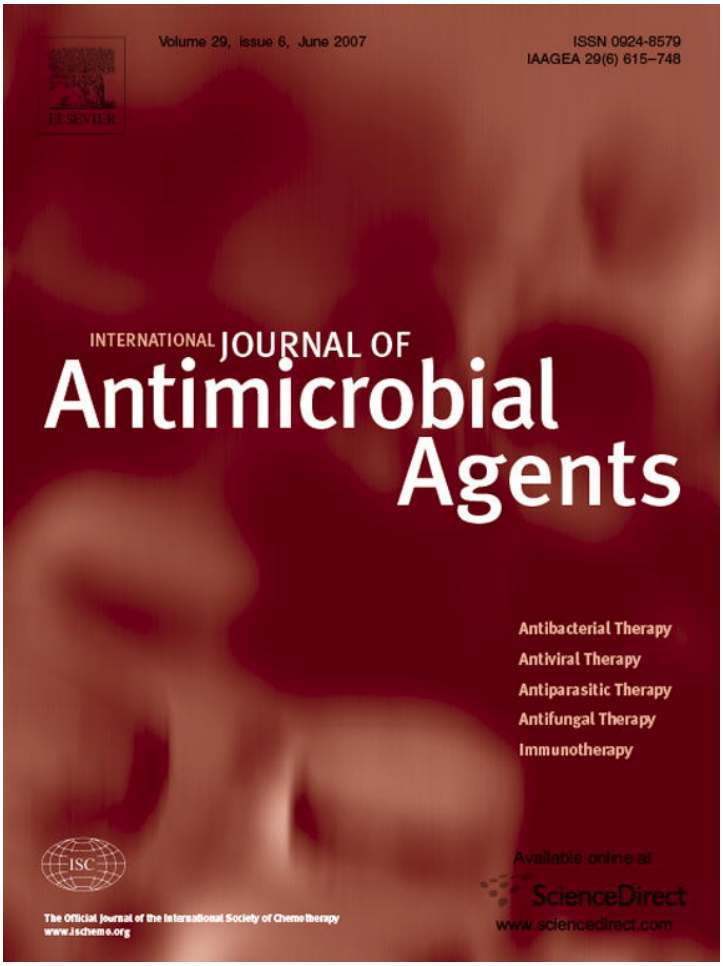

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Review

Leishmania spp.: proficiency of drug-resistant parasites

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Abstract

Leishmaniasis is a disease caused by at least 17 different species of protozoan *Leishmania* parasites and currently affects around 12 million people living mostly in tropical and subtropical areas. Failure to treat leishmaniasis successfully is often due to drug resistance. However, there are no cellular and molecular markers of chemoresistance against leishmanicidal drugs and the only reliable method for monitoring resistance of individual isolates is the in vitro amastigote/macrophage model. It is thus necessary to find cellular and molecular markers that can be used systematically to identify the drug-resistant phenotype of the infecting parasites. Until now, whether drug resistance in *Leishmania* compromises parasite proficiency, e.g. in terms of infectivity or metabolism, has not been systematically evaluated. Therefore, here we examine whether the physiological changes expressed by drug-resistant *Leishmania* reflect a modification of parasite vitality in drug-resistant compared with drug-sensitive parasites. Finally, the clinical implications of drug resistance in *Leishmania* are also discussed.

Keywords: Drug resistance; Proficiency; Glucose uptake; Infectivity; *Leishmania*; Metabolic adaptation

1. Introduction

Chemotherapy against leishmaniasis is based mainly on toxic pentavalent antimonials developed during the first half of the last century. During the last decade, alternative drugs have become available and registered for use in some countries [1]; however, most developments in chemotherapy against leishmaniasis have come from re-formulation and re-screening of already identified medicaments rather than from rational design of drugs. This is because both the biology of the parasite and the immunological response of the host are not yet well understood [2,3].

In fact, overall development of new drugs against leishmaniasis has been generally slow. For example, paramomycin was described in the mid 1980s as useful against cutaneous and visceral leishmaniasis, but entered the clinical phase only recently. Similarly, sitamaquine was first identified in the mid 1970s and since then only phase I/II trials have been completed, with various degrees of success [1,4]. Finally, the efficacy of miltefosine against leishmaniasis has been known since the mid 1980s, but it took until 2002 to enter the market as the drug of choice against visceral leishmaniasis in India and until 2005 against cutaneous leishmaniasis in Colombia [1,5]. However, the use of miltefosine is limited due to its teratogenicity, potential for resistance development and narrow therapeutic window [6–8].

Toxic pentavalent antimonials, which constitute the mainstay of treatment for leishmaniasis, have almost been abandoned in India owing to the lack of response of *Leishmania donovani* against glucantime and *N*-methyl glucamine, although they are still useful in the rest of the world [1,2]. A liposomal formulation of amphotericin B (AmBisome) has now moved to the forefront in India, even though amphotericin B and pentamidine are highly toxic and cause unpleasant side effects, including fever [9,10]. Chemotherapy of leishmaniasis thus requires close clinical control.

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Furthermore, antileishmanial treatments remain extremely expensive for countries in which the disease is endemic [11].

Unfortunately, the misuse of antileishmanial compounds has increased the frequency of chemoresistant cases as well as contributing to the rising incidence of human immunodeficiency virus (HIV)–*Leishmania* co-infections [8,12,13]. To date, no cellular or molecular markers of resistance to currently used antileishmanial drugs are available. Therefore, identification of such markers is not only desirable but also fundamental to prevent compounding of the current situation. Such a development would be especially important in settings in which patients have developed drug resistance and may have limited chemotherapeutic alternatives. It is also important to know whether drug resistance involves alterations in the parasite's capacity to grow, differentiate into metacyclics and then infect cells, highly developed skills that we define herein as proficiency, and also whether these changes are useful as molecular markers for drug resistance.

2. The problem of drug resistance in chemotherapy against *Leishmania*

Variations in the efficacy of drugs for the treatment of leishmaniasis are frequently a consequence of the immune status of the patient, the pharmacokinetic properties of the drug and the intrinsic differences in drug sensitivities of each *Leishmania* spp. [12,14]. Decreased drug sensitivity is defined as drug resistance when there is a decline in the effectiveness of a drug against a population of parasites previously susceptible to the compound. This definition assumes that the original susceptibility of the population is known; however, this is not always the case for *Leishmania* [15]. Indeed, resistance of *Leishmania* against a given drug may be either natural or acquired when the parasites are exposed to suboptimal drug doses [14,16].

In zoonotic diseases, such as most cases of cutaneous and visceral leishmaniasis caused by *Leishmania infantum* and *Leishmania chagasi*, respectively, the parasite primarily infects domestic mammalian hosts and only occasionally infects humans. The immediate consequence is that the time that a parasite population is exposed to a drug is insignificant unless the mammalian reservoir host is also treated, as has been the case for dogs infected with *L. infantum* [17,18]. For this reason, current knowledge of the epidemiology and transmission of leishmaniasis suggests that the spread of acquired drug resistance is not an important factor to be considered in cutaneous leishmaniasis, except in the anthroponotic foci of *Leishmania tropica* [14]. However, such drug resistance is becoming a problem in *L. infantum*-mediated leishmaniasis, especially in cases of drug abusers infected with the parasite, where transmission is from human to human by needle, and in *L. donovani*-mediated visceral leishmaniasis in Bihar, India, where 70% of cases do not respond to therapy [14,19,20]. Pentavalent antimonials were used worldwide for the treatment of leishmaniasis for more than 60 years without evidence of resistance. However, during the last 15 years increased clinical resistance has become worrisome, hitting particular patient groups hardest, such as those co-infected with visceral leishmaniasis and HIV. The main reason for the emergence of resistance is the widespread misuse of the drug [14]. Furthermore, resistance against pentavalent antimonials represents one of the most serious problems in the control of visceral leishmaniasis especially in areas such as Bihar state in India [16,21]. To date, the only reliable method for monitoring resistance of individual isolates is the use of the technically demanding in vitro amastigote/macrophage model [14]. For this reason, there is an urgent need to identify cellular and molecular markers of resistance that can both evaluate resistance development in relation to treatment outcome and are easy and cost effective to use.

3. The concept of cell proficiency

In the case of viruses, fitness has been defined as their ability to reproduce and infect successfully in a defined environment [22–24]. Herein we will define fitness as proficiency, i.e. the complex integrated skills that allow the organism to replicate and transmit the disease successfully. When a virus acquires drug resistance, it often pays a price in terms of reduced ability to replicate and cause disease [23]. The degree of impairment of viral replication varies widely in viral strains resistant to, for example, antiretroviral drugs. However, a broad variability in viral capacity to replicate also exists in wild-type viruses owing to the natural viral genetic polymorphisms present in each viral strain [23]. As a result, the deleterious effect of viral proficiency associated with acquisition of drug resistance is still unclear.

In the case of bacteria, antibiotic resistance is also often associated with reduced competitive ability against antibiotic-sensitive strains in the absence of antibiotics [25,26]. In diverse model systems, the cost paid to achieve resistance depends on the specific mutation conferring drug resistance and the strain's genetic background. Moreover, this 'cost' can be reversed by compensatory mutations [26].

In the case of parasites, a decrease in proficiency has also been a cause of debate. Good examples of this include drug-resistant members of the apicomplexan parasites such as *Toxoplasma gondii* and *Plasmodium falciparum* [27,28] as well as *Schistosoma mansoni* [29]. Isolates of *S. mansoni* that exhibit a decreased response to praziquantel also have a lower reproductive efficiency and a compromised ability to compete with other isolates [29]. A similar scenario has also been reported for *T. gondii* with a mutated dihydrofolate reductase gene [27]. In *Plasmodium chabaudi*, a pyrimethamine-resistant mutant has been found to grow more slowly in the presence of drug pressure than its drug-sensitive progenitor, but to grow faster when seeded in the absence of

drug [28]. This finding suggests that compensatory mutations allow the parasite to lead a successful life [30]. Compensatory mutations are also known to be present in other drug-resistant parasites, e.g. chloroquine-resistant plasmodia [28].

In the case of *Leishmania*, virulence is used as the foremost marker for proficiency, and a virulence marker is defined as one fundamental for the survival and pathogenesis of the parasite within the sandfly or mammalian host [31]. This definition may include markers whose loss results in a quantitative but not complete loss of virulence as well as those that may affect growth and differentiation in culture [31,32].

The concept of fitness (or proficiency) in *Leishmania* has been mainly related to programmed cell death as a mechanism to allow the survival of the one with the best skills to guarantee transmission to the next host [33]. However, trypanosomatids are able to adapt their energy metabolism easily and rapidly to cope better with a diverse range of stress conditions such as those imposed by drug pressure. However, whether the development of drug resistance in these parasites involves a 'cost' and implies a detrimental effect in their proficiency (in terms of infectivity or metabolism) has until now not been systematically evaluated.

4. Studying cell proficiency in drug-resistant *Leishmania*

Proficiency (or fitness) is best defined in the case of a virus by its capacity to replicate in growth condition experiments [22]; however, numerous reports have extended this definition to that of relative fitness to include changes in either the catalytic activity of key metabolic enzymes or the infectivity of the virions [24], or the metabolic impact of a specific mutation [28]. The predictive value of these potential markers is still under evaluation, but hopefully their use will establish general guidelines for clinical decisions in the case of patients suspected of harbouring drug-resistant viruses.

In the case of *Leishmania*, several changes in physiological parameters are associated with drug resistance and might serve as markers for parasite proficiency.

4.1. Phenotype of drug-resistant Leishmania

Overexpression of membrane-bound ATP-binding cassette (ABC) proteins is directly linked to drug resistance in *Leishmania* [14–16]. These transport systems modulate the efflux or intracellular trafficking of chemotherapeutic agents. Furthermore, the development of drug resistance in *Leishmania* is also related to other biochemical and physiological parameters [15]. Whether alteration in these physiological parameters represents a change in cell proficiency has not been systematically studied.

In addition to drug transport through the multidrug-resistant (MDR) P-glycoprotein or the multidrug-resistance associated protein (MRP), other mechanisms described so far involve fundamental physiological functions [15], for example the decreased expression of prominent virulent factors of the parasite (such as lipophosphoglycan, acid phosphatase and Meta-1 expression) [34–36], the decreased incorporation of folates and nucleosides (metabolites considered to be fundamental for parasite survival) [37,38], the considerable increase in conjugation and traffic of xenobiotics (trypanothione and Cyb expression) [39–41] and the change in the amount and activity of enzymes for intracellular metabolism (dihydrofolate reductase–thymidylate synthetase, *N*-acetylglucosamine-1-transferase and pterin transferase) [42]. Finally, there are alterations in events related to metacyclogenesis and infectivity, such as membrane fluidity, lectin agglutination, cell shape and promastigote/amastigote differentiation (tubulin phosphorylation) [36,43,44]. Using these physiological changes as a basis, we discuss how they may help to establish reliable methods for monitoring resistance of isolates. Fig. 1 summarises the physiological changes that may be related to the 'cost' made by *Leishmania* to achieve drug resistance.

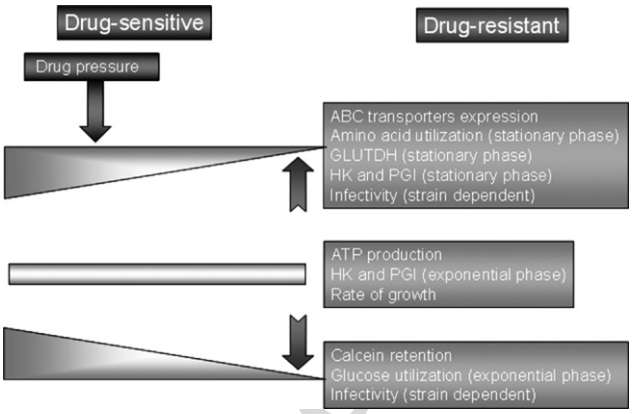

Fig. 1. Physiological changes that may be related to cell proficiency in drug-resistant *Leishmania*. Drug pressure induces drug resistance in *Leishmania* and triggers a range of changes (see text for details). ABC, ATP-binding cassette; GLUTDH, glutamate dehydrogenase; HK, hexokinase; PGI, phosphoglucose isomerase.

4.2. Calcein retention in drug-resistant Leishmania

Cellular events that occur along with *Leishmania* drug resistance include the overexpression of ABC proteins that modulate the efflux or intracellular trafficking of chemotherapeutic agents (Fig. 1) [14–16]. Calcein acetoxymethyl ester (CAL-AM), the hydrophobic derivative of calcein, can be expelled from resistant cells by either the MDR or MRP mechanism. Cellular accumulation of this fluorophore and its inhibition by specific compounds such as verapamil provide a quantitative measure of cell transport activity through ABC proteins and have been proposed to represent a proof of concept of a strain being resistant. However, energy-dependent CAL-AM efflux systems can be constitutively expressed in *Leishmania* [45,46] and therefore do not necessarily validate

the drug-resistant phenotype of a *Leishmania* strain. These studies were, however, performed with laboratory strains and do not necessarily represent what happens in vivo. Since the calcein technique could represent a routine clinical laboratory method, it would be interesting to assess its potential use as a cellular marker for identification of the drug-resistant phenotype in patient isolates.

4.3. Growth kinetics of drug-resistant Leishmania

As mentioned previously, the definition of cell proficiency includes its capacity to replicate in growth condition experiments [22]. This definition overlaps in *Leishmania* with the notion of virulence; in this parasite, the concepts of differentiation and virulence are linked with specific timings within the kinetics of growth and are determined by different variables, among them the cell density. Therefore, in *Leishmania* it is important to know whether changes in growth kinetics represent a difference in cell proficiency and whether there is an association between these two parameters and drug resistance. For example, in the case of *Leishmania amazonensis*, the growth kinetics of drug-sensitive parasites differs slightly from that of drug-resistant parasites grown under pressure of the drug [46,47] (Fig. 1). However, when cultivated in the absence of drug, the drug-resistant parasites grow faster than the wild-type strains, meaning that they have a higher replication capacity [22]. As for viruses [22], *Leishmania* parasites exhibit a broad range of replication capacities, even in the absence of genotypic or phenotypic markers for resistance. This means that changes in replication capacity may be an independent characteristic rather than a proxy marker for drug resistance and that growth kinetic assays cannot be taken as a measure of parasite potency or physical condition. However, similar to HIV-infected patients, it is not yet known in *Leishmania* whether there is an association between viral proficiency (or fitness) or replication capacity and a patient's status of 'non-progressor' [22]. Hence, it would be interesting to analyse whether in *Leishmania*-infected patients there is an association between immune-competent cell counts (in this case macrophages) and lower replication capacity of the parasites and, if so, to determine its correlation with therapy failure and infection by drug-resistant parasites.

4.4. Enzyme activity of drug-resistant Leishmania

Drug resistance is a useful tool for exploring metabolic pathways and potential parasite responses to current or prospective chemotherapy [28]. Additionally, many metabolic pathways in *Leishmania* are intimately involved in parasite virulence, beyond their role in viability. This is the case for the enzymes pteridine reductase and trypanothione reductase, for example [15]. However, whether changes in these or other metabolic pathways can predict changes in cell proficiency associated with drug resistance is not yet known. For example, it is not clear whether the correlation between enzyme activity and metabolite flow through glycolysis is a good marker for cell potency [28] (Fig. 1). Interestingly, exponential-phase resistant parasites exhibit decreased use of glucose as an energy substrate, decreased glucose uptake and decreased glucose transporter expression. However, compared with drug-sensitive cells, stationary-phase resistant parasites display increased use of amino acids as an energy substrate and increased activity of the enzymes hexokinase, phosphoglucose isomerase and especially NAD^+-linked glutamate dehydrogenase (GlutDH) (Fig. 1) [46–49].

These results may indicate that drug resistance in stationary-phase *Leishmania* activates the utilisation of oxidative phosphorylation, since the NAD^+-regulated GlutDH is involved in directing amino-acid-derived metabolites into the Krebs cycle [50]. Interestingly, production of ATP is similar in drug-sensitive and -resistant parasites (Fig. 1) [51]. This observation may also imply that in exponentially growing *Leishmania*, the activities of enzymes located at the beginning of the glycolytic pathway do not depend on the velocity of glucose uptake and therefore are not related to a change in cell proficiency.

Since these results suggest that continued drug pressure induces a metabolic adaptation that allows the parasite to select metabolic pathways to compensate for the primary defects produced by the drug pressure, they may mean that changes in these metabolic pathways cannot predict changes in proficiency associated with drug resistance. However, it would be interesting to analyse whether, in *Leishmania* isolates obtained from infected patients, there is a correlation between therapy failure or infection by drug-resistant parasites and a slower rate of glucose uptake and of glucose turnover during the parasite's exponential growth phase.

4.5. Metacyclogenesis in drug-resistant Leishmania

Metacyclogenesis is a lifecycle differentiation process during which co-ordinated changes in metabolism and morphology occur to effect transformation from the non-infective proliferative promastigote to the non-proliferative infective amastigote. This differentiation is marked by cell cycle arrest and acquisition of infectivity [52,53]. Sandfly stages are closely resembled in vitro by cultured log phase promastigotes. Upon entry into stationary phase, they undergo differentiation into a form that closely resembles metacyclic (infective) promastigotes. The ability of the parasite to grow in vitro makes it possible to mimic relevant parasite differentiation stages and may be useful to answer questions such as whether drug resistance alters the probability of differentiation and of being infective.

There is evidence to suggest that drug resistance may be associated with a decrease in the infectivity [proficiency?] of some strains (Fig. 1). Indeed, this is the case for amphotericin-resistant *Leishmania mexicana* [54], *L. mexicana amazonensis* resistant to ABC transporter blockers [34,43], glucantime-resistant *Leishmania* (*Viannia*) *guyanensis* [35] and ricin-resistant *Leishmania major* [55,56]. In contrast, *L. mexicana amazonensis* resistant to tunicamycin,

a drug not relevant for the treatment of leishmaniasis, shows an increased or unchanged virulence compared with the sensitive strain [57–60]. Whether decreased infectivity is a consequence of lower cell proficiency with few morphological changes is still unknown.

Metacyclic parasites should possess short, narrow cell bodies [52,53]. However, stationary-phase drug-resistant cells have greater length and area than drug-sensitive cells [43]. Additionally, stationary-phase drug-resistant cells are more sensitive to human serum complement than sensitive cells, suggesting a modification in the expression of surface membrane carbohydrates [43]. Finally, stationary-phase drug-resistant parasites express less Meta-1 than drug-sensitive cells [43]. This marker is the product of the *meta-1* gene, originally described in *L. major* [61]; it is highly conserved both in Old and New World parasites and is predominantly expressed in metacyclic promastigotes of *Leishmania* [61,62]. As previously mentioned, the only reliable current method for monitoring resistance of isolates is the technically demanding in vitro amastigote/macrophage model [14]. Hence, it is of extreme importance to evaluate whether cellular and molecular markers such as those just described can be taken as a measure of parasite proficiency, correlate with the expression of drug resistance in patient isolates and can be cheaply implemented for determining treatment outcome.

5. Perspective

Continued therapy upon emergence of drug resistance may be viewed as maintenance of a less healthy variant and as a means to reduce the parasitic load in the absence of actual drug-mediated inhibition. However, one implication of this observation is that parasite populations might evolve new levels of virulence in response to medical interventions such as drugs. Intentional selection of drug-resistant variants with reduced proficiency may be proposed as an alternative therapy. However, a less competent drug-resistant parasite could evolve as a competent isolate, i.e. one that is stable and upon removal of drug pressure can be transmitted to new human recipients. It should also be borne in mind that in vitro estimates of growth replication capacity cannot be easily extrapolated to in vivo situations. Also, more studies are necessary to establish a validated protocol to measure cell proficiency both in the presence and absence of drugs. Such standardisation is essential. However, the continual evolution of resistant strains might lead to compensatory mutations that eventually result in return to normal cell potency. In the search for these markers, the phenotype of individual species subjected to drug pressure must be monitored. In particular, in isolates obtained from patients it is necessary to evaluate characteristics such as metabolite usage and enzymatic pathway fluxes, in parallel with resistance selection and infectivity. Their prognostic value for treatment outcome might be extremely useful.

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