

Review Article

Drug Resistance in Visceral Leishmaniasis

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Visceral leishmaniasis remains a public health problem worldwide. This illness was included by the World Health Organization in the list of neglected tropical diseases targeted for elimination by 2015. The widespread emergence of resistance to pentavalent antimonials in India where half cases occur globally and the unavailability of a vaccine in clinical use constitute major obstacles in achieving this goal. The last decade new antileishmanials became available, including the oral agent miltefosine. However, in poor endemic countries their wide use was curtailed because of the high costs, and also due to concerns of toxicity and emergence of resistance. Various mechanisms of antileishmanial resistance were identified recently in field isolates. Their elucidation will boost the design of new drugs and the molecular surveillance of resistance. Combination regimens should be evaluated in large trials. Overall, the development of antileishmanials has been generally slow; new drugs are needed. In order to control visceral leishmaniasis worldwide, treatment advances should become affordable in the poorest countries, where they are needed most.

1. Introduction

Visceral leishmaniasis (VL; also known as kala azar) is a protozoan systemic infection, which is almost always fatal if left untreated. This illness is endemic in several tropical and subtropical regions and in the Mediterranean basin. The estimated annual global burden of VL is 500 000 new cases and more than 50 000 deaths, of which 90% occur just in five countries—India, Bangladesh, Nepal, Sudan, and Brazil [1]. VL is transmitted through hematophagous sandflies and is caused by *Leishmania donovani* in the Indian subcontinent, Asia, and Africa, *L. infantum* in the Mediterranean basin, and *L. chagasi* in South America. After an incubation period of several months, typical VL manifests with intermittent fever, weight loss, massive hepatosplenomegaly, and progressive deterioration of the host; hemorrhages and edemas may develop late in the course [2–4]. Leishmaniasis was selected by the World Health Organization for elimination by 2015, along with other neglected tropical diseases [5]. Since there is no antileishmanial vaccine in clinical use, control of VL relies almost exclusively on chemotherapy.

For almost seven decades pentavalent antimonials constituted the standard antileishmanial treatment worldwide,

however the last 15 years their clinical value was jeopardized due to the widespread emergence of resistance to these agents in Bihar, India, where half of VL cases occur globally [6]. The last decade novel formulations of conventional antileishmanials as well as new drugs, including the oral agent miltefosine, became available or are under investigation. In practice, however, their wide use in poor countries is hampered mainly due to high costs and also due to concerns of toxicity and emergence of resistance [6]. In response to concerns about preserving the currently available antileishmanials, especially in regions with anthroponotic parasite transmission, there is growing interest on combination regimens. Control of VL in poor countries is further compromised by the emergence of human immunodeficiency virus (HIV)-VL-coinfection [7]. This article will review recent publications on antileishmanial drugs, with emphasis on resistance issues. Strategies to preserve the activity of currently available drugs will be addressed.

2. Pathogenesis and Immune Response

Leishmaniasis are obligatory intracellular protozoan parasites. The parasites remain within their vectors as extracellular promastigotes [8]. Following sandfly bite, neutrophils migrate

locally and capture the parasites, however the latter have the ability to escape and subsequently invade the macrophages of the skin, where they differentiate and replicate as amastigotes [9, 10]. From there, parasites disseminate and invade additional macrophages of the reticulo-endothelial system, and finally infiltrate the bone marrow, liver, and spleen [8]. VL should be regarded as a state of long-term parasitism, since leishmanias are not eradicated completely but rather remain in skin macrophages for lifetime, even after successful treatment in hosts with intact T-cell immune responses. In skin, leishmanias act as a reservoir for the potential relapse of symptomatic VL. The risk for relapse increases when T-cell immune responses are impaired and irrespectively of prior antileishmanial treatment, as noted in HIV-infected patients [7, 11, 12]. Relapses usually peak 6–12 months after treatment.

Following *Leishmania* infection, host immune responses are elicited. Immune responses are characterized by a mixed T-helper cell-type 1 (Th1) and Th2 response, the production of cytokines, and the activation of macrophages [13–15]. High levels of specific antibodies are also detected however their exact role remains unclear [8]. Recent evidence indicates that the first weeks following infection, neutrophils play a significant role in the killing of parasites and the development of a protective Th1 immune response [9, 10].

The immunologic mechanisms that underlie the resolution of infection or the progression and systemic dissemination of leishmanias have not been elucidated completely so far. Following infection, T-cell-dependent immune responses are elicited in an integrated fashion. Interleukin 12 (IL12) promotes cell-mediated immunity. Activated CD4 T cells are recruited to cutaneous or visceral sites of infection and direct the local inflammatory responses. CD4 T-cell responses are associated with the interferon (IFN)- γ -induced macrophage activation through participation of cytokines, mainly IL12 and also IL2 and tumor necrosis factor, and the intracellular parasite killing by activated macrophages [8]. IL4 also plays an important role in effective antileishmanial chemotherapy, which appears to be modulated by IFN- γ -production [16]. Deactivation of macrophages, suppression of Th1 responses, and dissemination of leishmanial infection are induced by IL10 [14]. Increased IL10 levels have been detected repeatedly in human VL and are considered crucial in uncontrolled leishmanial infection [13, 14]. Targeting IL10 has been associated with activation of Th1 responses and parasite killing, whereas IL10 suppression constituted a critical step in vaccine-mediated immunotherapy [17]. Most data on cellular immune responses and cytokines have been observed in murine models; similar results have been found in humans.

3. Antimonials

Although pentavalent antimonials (meglumine antimoniate and sodium stibogluconate) are in clinical use for several decades, there are aspects on their mechanism of action that remain unclear. It is generally accepted that pentavalent antimonials (SbV) are the prodrug, and that they

should convert to trivalent antimonials (SbIII) in order to demonstrate their antileishmanial activity [18–20]. Recent evidence indicates that antimonials kill leishmanias by a process of apoptosis [20]. Thiol metabolism is critical in their mechanism of action. Trypanothione is the major thiol in leishmanias. SbIII inhibits trypanothione reductase in vitro, inducing the loss of intracellular thiols and a lethal imbalance in thiol homeostasis, leading to accumulation of reactive oxygen species [20–22]. In order for antimonials to exert their action, an almost intact immune system of the host is required.

Initially antimonials were given at 10 mg/kg for 6–10 days with >90% cure rates, however after the first treatment failures occurred in India two decades ago, higher doses and prolonged schemes (up to 20 mg/kg for 30 days) were introduced gradually and in parallel with the increasing rates of antimony unresponsiveness [23]. However, the dose escalation policy did not prevent further emergence of resistance, but rather selected resistant parasites. During the last decade, antimonial resistance and therapeutic failures reached epidemic dimensions in Bihar, India; nowadays, up to 60% of newly diagnosed VL cases in this area do not respond to antimonials [23]. Inadequate treatment in terms of dosing and duration, and poor compliance promote the widespread antimonial resistance in India. In this country, the high incidence rate of unresponsiveness to antimonials is further sustained by the anthroponotic transmission of leishmanial infection, which increases the chances for the rapid spread of resistant parasites among humans once they emerge [24, 25]. Low rates of antimonial resistance have been reported in Sudan also [26]. Pentavalent antimonials were abandoned in India, however they remain the first treatment choice in most VL-endemic areas in the rest of the world, with efficacy rates exceeding 90%–95% and low case fatality and relapse rates [2–4, 27]. Low cost is their main advantage. Disadvantages include intramuscular administration, prolonged treatment, and transient, but occasionally life-threatening adverse effects, such as cardiac arrhythmias, increased hepatic transaminases, pancreatitis, and pneumonitis [2–4, 23].

While several experimental studies on antimonial resistance have been conducted with parasite mutants selected in vitro using step-wise increasing drug concentrations, resistance mechanisms in field parasites have not been elucidated in details. Mechanisms of in vitro antimonial resistance may differ from mechanisms in field isolates [28]. Similarly, in vitro unresponsiveness does not necessarily translate to clinical resistance [29]. Reduction of drug concentration within the parasite, either by decreasing drug uptake or by increasing efflux/sequestration of the drug, constitutes the primary mechanism of antimonial resistance; other potential resistance mechanisms include inhibition of drug activation, inactivation of active drug, and gene amplification [18, 20, 28, 30–32].

Thiol metabolism possesses a key role in both clinical and laboratory-generated resistance mechanisms. It has been found that elevated intracellular thiol levels and overexpression of trypanothione peroxidase are associated with high levels of SbIII resistance [22, 31, 33]. However, it appears that

more than one step in thiol metabolism should be impaired in order for resistance to emerge, indicating that antimonial resistance is multifactorial. In natural antimonial resistance, the impaired thiol metabolism results in inhibition of SbV activation and decreased uptake of the active form SbIII by amastigotes; these processes are accomplished by the lower expression of the genes γ -glutamylcysteine synthetase, ornithine decarboxylase, and aquaglyceroporin 1, which are involved in the metabolisms of glutathione and trypanthione, and uptake of SbIII, respectively [18, 19, 28]. It has been suggested that decreasing the intracellular thiol concentrations through thiol depletors may increase the leishmanicidal action of drugs and thus reverse parasite resistance [33].

Overexpression of the membrane-bound ATP-binding cassette (ABC) transporters on the surfaces of leishmanias is another mechanism of antimonial resistance. In addition to leishmanias, this transport system modulates the efflux and intracellular accumulation of various drugs and thus resistance in other parasites (e.g., *Plasmodium* spp.) and also in cancer cells. Overexpression of ABC transporters concerns laboratory-derived and in-field resistant parasites [31, 34]. It has been found that, in contrast to infection with Sb-sensitive *L. donovani* isolates, infection with Sb-resistant *L. donovani* isolates upregulates the multidrug resistance-associated protein 1 (MRP1) and the permeability glycoprotein (P-gp) in host cells, thus inhibiting intracellular drug accumulation by decreasing antimony influx [31, 34, 35]. In animal models, inhibition of the proteins MRP1 and P-gp by lovastatin reverses their action on drug accumulation, and allows them to escape a fatal outcome [35]. These results indicate that lovastatin, which can inhibit P-gp and MRP1, might be beneficial for reverting Sb resistance in VL [35]. Flavonoid dimers are also known to reverse antimonial resistance in leishmanias in vitro by inhibiting ABC transporters and increasing the intracellular accumulation of the drug [36]. These findings should be confirmed in animal models.

In conclusion, the overall phenomenon of antimonial resistance is multifactorial. Several mechanisms of resistance to antimonials have been detected among clinical leishmanial isolates. However, the modes of emergence and spread of antimonial resistance in field remain largely unknown. A monoclonal or oligoclonal distribution of resistant parasites would be expected, given the anthroponotic nature of leishmanial transmission in the Indian subcontinent. However, a study of 13 Sb-resistant and 11 Sb-sensitive *L. donovani* clinical isolates collected from Nepal using DNA fingerprinting methods in a population genetics approach revealed a polyclonal distribution of resistant isolates and three major clusters, each containing both sensitive and resistant isolates [37]. Analysis of isolates of paired samples collected from the same patients before treatment and after treatment failure showed primary as well as acquired resistance [37]. Based on these findings, the hypothesis of independent events of emergence of drug resistance appears likely, which suggests a pleiotropic answer of leishmanias to drug pressure, as indicated by the various existing mechanisms of antimonial resistance. High genomic variability among *L. donovani* clinical isolates from India was also found with the use of amplified fragment length polymorphism, suggesting that

various point genetic rearrangements provide the frame for the transition of a parasite from sensitive to resistant [38].

4. Amphotericin-B and Its Lipid Formulations

Conventional amphotericin B has been used as a second-line treatment for VL since the 1960s. This drug exhibits an excellent antileishmanial activity with >90%–95% cure rates in Indian VL cases. Unresponsiveness and relapses occur rarely, except among HIV-infected patients [3, 11, 12]. In this population, secondary episodes of VL are common and are attributed mainly to relapse but also to reinfection [11]. A recent study failed to disclose decreased susceptibility among leishmanias collected from HIV-infected patients during repeated VL episodes (mean follow-up period: 35.6 months; range: 3–137 months), despite repeated courses of amphotericin B; these data indicate that amphotericin B will remain a very useful drug for the treatment or secondary prophylaxis in this group of patients, even after repeated use [11].

The routine scheme of conventional amphotericin B is 1/mg/kg administered on alternate days for a total of 30 days, however, a recent study in India showed 96% cure rates with a dose of 0.75 mg/kg/day for 15 days [6]. Major disadvantages of conventional amphotericin B are its prolonged administration and the frequent adverse effects, such as infusion-related fever and chills, nephrotoxicity, and hypokalemia, which necessitate administration in hospital [6]. Conventional amphotericin B is used extensively in India for cases unresponsive to antimonials or even as a first line drug. However, outside India this drug does not offer any advantage over pentavalent antimonials.

Lipid formulations of amphotericin B improved highly the safety profile of this drug. Lipid formulations are taken selectively by the reticulo-endothelial system, and exhibit a highly localized enhanced antileishmanial action. There are three lipid formulations of amphotericin B: liposomal amphotericin B, amphotericin B lipid complex, and amphotericin B cholesterol dispersion. Currently, liposomal formulations of amphotericin B are the first treatment choice in southern Europe endemic countries as well as in other developed countries, because of their rapid and up to 100% cure rates with 3–5 days schemes, improved convenience for the patient, and reduction of health-care costs [27, 39, 40]. However, in poor countries even short courses of liposomal formulations are unaffordable, and the selection of antileishmanial treatment turns more to a question of cost than of efficacy or toxicity [6, 27]. The use of nanoparticles and microspheres for the delivery of conventional amphotericin B also increased its efficacy against experimental VL [41–43]. Similar results have been reported with the heat-induced reformulation of amphotericin B [44].

5. Miltefosine

Miltefosine (hexadecylphosphocholine) is the first orally administered drug for VL and the latest to enter the market. This agent is associated with high efficacy rates,

including cases unresponsive to antimonials [45, 46]. In a phase IV multicenter trial in India of 1132 adults and children with VL treated with miltefosine, cure rates were 82% per intention-to-treat analysis and 95% per protocol analysis [47]. In this study, 3% of patients developed adverse effects, mainly gastrointestinal toxicity, and elevated hepatic transaminases and creatinine [47]. So far, miltefosine is licenced in India, Germany, and Colombia. The scheme of miltefosine is 100 mg/kg/day for 28 days in adults weighing ≥ 50 kg, 50 mg/kg/day in adults < 50 kg, and 2.5 mg/kg/day in children (maximum dose: 100 mg/day). Major concerns for the wide use of miltefosine include its teratogenic potential and its long half-life (approximately 150 hours) which may facilitate the emergence of resistance. Miltefosine is strictly forbidden in women of child-bearing age who may become pregnant up to two months following drug discontinuation. In India miltefosine is available over the counter, a fact that may expose this drug to misuse and emergence of resistance. Once generated, resistant parasites could spread rapidly, endangering the life span of miltefosine in a country where it is needed most.

The exact antileishmanial mechanism of miltefosine remains largely unknown. The intracellular accumulation of the drug appears to be the critical step for its action. The intracellular accumulation of miltefosine includes the following steps: binding to plasma membrane, internalization in the parasite cell (two proteins, the miltefosine transporter LdMT and its beta subunit LdRos3, are the most significant), and intracellular targeting and metabolism [48]. It has been found that miltefosine induces an apoptosis-like cell death in *L. donovani*, by producing numerous defects [48]. Miltefosine also induces several immunologic and inflammatory effects on macrophages. In animal models, miltefosine does not require T-cell-dependent immune mechanisms in order to act, indicating that this agent can be used in T-cell-deficient patients [12, 48]. Recently, it was found that miltefosine enhanced IFN- γ receptors and thus IFN- γ responsiveness in *L. donovani*-infected macrophages; in the same model, miltefosine induced an IL-12-dependent Th1 response and reversed the Th2 response to Th1 response [49].

Resistance to miltefosine may emerge easily during treatment due to single point mutations [50, 51]. Decrease in drug accumulation is the common denominator in all miltefosine resistant *Leishmania* lines studied to date, and this could be achieved through decreased uptake, increased efflux, faster metabolism, or altered plasma membrane permeability; the first two mechanisms have been already described in models of experimental miltefosine resistance [48, 50]. Two proteins, miltefosine transporter LdMT and its specific beta subunit LdRos3, form part of the miltefosine translocation machinery at the parasite plasma membrane, and are required for miltefosine uptake [48]. Experimental mutations at LdMT or LdRos3 rendered the parasites remarkably less sensitive to miltefosine, and this resistance persisted in vivo; cross-resistance with other antileishmanials was not detected [48, 50]. The overexpression of ABC transporters is another mechanism for acquisition of miltefosine resistance, through reduction of the drug intracellular

accumulation [48, 52]. Recently, a novel flavonoid derivative was designed and it was shown that the use of suboptimal doses in order to overcome the overexpression of LtrMDR1 (a P-glucoprotein-like transporter belonging to the ATP-binding cassette superfamily) was associated with a four-fold increase of intracellular miltefosine accumulation in the resistant *Leishmania* lines [53]. Furthermore, modifications in lipid compositions of membranes and sterol biosynthesis have been detected in miltefosine-resistant *L. donovani* promastigotes [54]. Since membrane fluidity and permeability are influenced by lipid composition, their modification may affect drug-membrane interactions [54]. A case of a healthy patient with VL who relapsed 10 months after successful treatment with miltefosine for 28 days was reported recently [55].

6. Paromomycin

Paromomycin (aminosidine) is an aminoglycoside with antileishmanial activity. In a phase III study of VL in India, this drug was associated with 94.6% cure rates, similar to amphotericin B [56]. Adverse effects were more frequent in the paromomycin-treated group compared with the amphotericin B-treated group (6% versus 2%, resp.); paromomycin-related adverse effects included elevated hepatic transaminases, ototoxicity, and pain at injection-site [56]. Currently, paromomycin is under phase IV clinical trials. Paromomycin is inexpensive but requires daily intramuscular injections for 21 days [6].

Paromomycin inhibits protein synthesis and modifies membrane fluidity and permeability. An in vitro study showed that following a 72-hour exposure to *L. donovani* promastigotes and amastigotes to paromomycin, the mitochondrial potential was decreased, which indicates that mitochondria are the targets of the drug [57]. In laboratory-derived resistant parasites developed through serial-passage increasing-drug concentrations, paromomycin uptake was decreased compared to the wild-type parasite, in association with inhibition of protein synthesis; no cross-resistance with other antimonial agents was detected [57]. Since paromomycin is an aminoglycoside, it is possible that resistance will emerge rapidly if used as monotherapy.

7. Combination Regimens

The rationale for using combination regimens with different resistance mechanisms over monotherapy relies on the expected enhanced efficacy (through synergy or additive activity without drug interaction), shorter treatment duration, less toxicity, improved compliance, reduced likelihood of emergence of resistance, and reduced costs. A combination policy for VL is supported by the fact that antileishmanial drugs belong to different chemical classes. Recent studies have investigated this option. In a retrospective study conducted among Sudanese patients with VL, it was found that combination of sodium stibogluconate and paromomycin administered for 17 days was associated with higher cure and survival rates compared to sodium stibogluconate

monotherapy administered for 30 days (44%–86% lower odds of death in the combination group) [58]. Combinations of miltefosine with amphotericin B, paromomycin or pentavalent antimonials have been evaluated in an in vivo model and revealed that the combinations of miltefosine with amphotericin B or paromomycin were efficacious [59]. These preliminary data justified a recent study in Bihar, India, comparing 5 mg/kg of liposomal amphotericin B administered once (group A; 45 patients), 5 mg/kg of liposomal amphotericin B administered once plus miltefosine for either 10 days (group B; 46 patients) or 14 days (group C; 45 patients), 3.75 mg/kg of liposomal amphotericin B administered once plus miltefosine for 14 days (group D; 45 patients), and 5 mg/kg of liposomal amphotericin B administered once followed by miltefosine for 7 days (group E; 45 patients); in this study, similar final cure rates (91%–98%) were noted in all treatment groups [60]. These data indicate that a single dose of liposomal amphotericin B followed by 7–14 days of miltefosine is active against Indian VL [60]. In this study, all patients were treated in an outpatient setting. Large, randomized-controlled trials are required before adaptation of combination regimens.

Several combination regimens with investigational agents have been tested in vitro and in animal models [61]. The plant-derived immunostimulant agent picroliv has no antileishmanial activity, however when administered with half-dose miltefosine increases significantly the activity of the later [62]. The combination of verapamil (a calcium channel blocker) and diperoxovanadate (a potent antileishmanial agent) with sodium antimony gluconate reversed the in vitro antimonial resistance among clinical *L. donovani* isolates [63, 64]. Diperoxovanadate also demonstrated immunomodulating effects by increasing IFN and decreasing IL-10 [64]. These combinations deserve further testing in VL cases unresponsive to antimonials.

8. Strategies to Preserve the Efficacy of Currently Available Antileishmanials

In addition to intrinsic pharmacologic features, there is a number of human parameters that may favor the emergence and spread of leishmanial resistance. These include poor compliance, expensive treatment, availability of antileishmanial drugs over the counter, and limited access to health-care facilities for early diagnosis and treatment. Given the current situation of the widespread emergence of antimonial resistance in India, there is growing concern to preserve the efficacy of novel antileishmanials. Such a strategy should focus on the following axons.

- (1) Treatment of VL should be based on guidelines for prompt diagnosis, selection of first-line drugs, management of cases unresponsive to antimonials, and HIV-coinfected cases. A recent study of Indian VL cases revealed that a strategy of treatment with antimonials (first choice) or amphotericin B (second choice), based on culture and susceptibility results, compared with an empiric treatment strategy, was associated with higher cure rates (86.21% versus

35.71%), and reduced expenses, duration of hospitalization, and likely period of spread of parasites in the community [65].

- (2) In order to enhance compliance, directly observed therapy for antileishmanials should be implemented, like in tuberculosis control programs.
- (3) VL cases should be treated early in order to avoid further transmission of resistant parasites in the community.
- (4) Distribution and clinical response of antileishmanials should be monitored.
- (5) Antileishmanial treatment should be provided free-of-charge through the health-care system.
- (6) The emergence and spread of antileishmanial resistance should be monitored.
- (7) The efficacy and safety of combination regimens should be evaluated in large trials.

9. Conclusions

The control of VL globally is challenged by the widespread emergence of antimonial resistance in India. The last decade new formulations of conventional antileishmanial drugs as well as new agents became available. The wide use of the oral agent miltefosine was hampered by the potential for teratogenicity and emergence of resistance. Combination regimens should be evaluated in large trials. The last years several mechanisms of in field antileishmanial resistance were identified. Understanding their molecular and biochemical characteristics will lead the design of new drugs and also the molecular surveillance of resistance. In order not to jeopardize the life span of available antileishmanials, their delivery, clinical response, and resistance should be monitored. Overall the development of antileishmanials has been generally slow; new drugs are needed.

Author's Statement

The findings and opinions in this review are those of the author and do not necessarily represent those of the Hellenic Center for Disease Control and Prevention.

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