RECENT STATUS OF ACCESSIBLE DRUGS RESISTANCE IN VISCERAL LEISHMANIASIS

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ABSTRACT
Visceral leishmaniasis residue a communal fitness trouble worldwide and it is form in which the internal organs are pretentious. Though, the mainly stern form visceral leishmaniasis can be lethal if left untreated. Resistance to pentavalent antimonials, which have been the suggested drugs to treat visceral leishmainiasis, is currently prevalent in Indian subcontinents. The unattainability of a vaccine in medical use constitutes main obstructions in accomplishing this target. Novel drug formulations similar to amphotericin B, its lipid formulations, and miltefosine have exposed immense usefulness to treat visceral leishmaniasis however their elevated price and therapeutic problems boundary their efficacy. Diverse machineries of antileishmanial resistance were identified currently in meadow segregate. Their illumination will enhance the design of novel drugs and the molecular observation of resistance. Amalgamation regimens should be assessed in bulky trials. Taken as a whole, the progress of antileishmanials has been usually sluggish; novel drugs are required. In regulate to control visceral leishmaniasis worldwide and treatment advances must become reasonable where they are needed mainly. Despite momentous advancement of leishmanial investigate through final few decades, detection and characterization of novel drugs and drug goals are far away from adequate.

KEY WORDS: Drug Resistance, Visceral Leishmaniasis, Pentavalent Antimonials, Amphotericin B, Miltefosine,
Introduction

Visceral leishmaniasis (VL) also known as ‘Kala-azar’ is caused by the protozoan parasite *Leishmania donovani* (LD), and is widening its bottom in diverse parts of the worldwide (1, 2). With an estimated 500,000 new cases of VL and 59,000 annual deaths, VL is second only to malaria in annual worldwide fatalities due to protozoal infections (3, 4). Over 90% of VL cases occur in six countries: Bangladesh, India, Nepal, Sudan, Ethiopia, and Brazil (5). The main factors driving VL incidence include migration, urbanization, lack of vector and/or reservoir control measures, opportunistic co-infections, and civil war (3, 5). In India VL reaches annual incidence rates of 2.5/1,000 person in highly endemic areas and prevalence of *L. donovani* infection based on serological evidence is currently estimated at 18% (6). Leishmaniasis consists of a complex of vector borne diseases caused by more than 20 species of the protozoan genus Leishmania and is transmitted by sand fly vectors (7). Two species of leishmaniasis are known to give rise to visceral form of the disease. Species commonly found in East Africa and Indian subcontinent is *L. donovani* and that found in Europe, North Africa and Latin America is *L. infantum*, also known as *L. chagasi*. Natural transmission may be zoonotic or anthroponotic by the bite of a phlebotomine sandfly species of the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World). The disease may be sporadic, endemic or epidemic. About 500 species of 6 genera of female ‘*Phlebotomus’ are suspected or proven as vectors transmitting parasites from animal to animal, animal to man and man to man. Leishmaniasis was selected by the World Health Organization for elimination by 2015, along with other neglected tropical diseases (8). 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 worldwide (9). Cases of VL along with HIV have also been reported by WHO has an emerging and intricate problem (10). People with HIV infection are at higher risk of contracting the diseases if they live in or travel to endemic regions. It is currently estimated that 25-70% of adult visceral leishmaniasis cases are related to HIV, and 1.5-9% of AIDS cases suffer from newly acquired or re-activated visceral leishmaniasis (11). The current situation for the chemotherapy of leishmaniasis is more promising than it has been for several decades with both novel drugs and new formulations of old drugs either recently approved or in clinical trial (Table 1) (12,13). In recent years four new potential therapies have been introduced for visceral leishmaniasis (Table 1). These include an amphotericin B liposome formulation registered in the United States and Europe (AmBisome) (14, 15); oral miltefosine (16) which has been registered in India and is now in phase IV trial; aparenteral formulation faminosidine (paromomycin) (17) currently completing phase III clinical trials in India (www.iowh.org) and on trial in East Africa (www.dndi.org); and oral sitamaquine, which has completed phase II trials in India, Kenya, and Brazil (18-20) and is in development with GlaxoSmithKline (http://science.gsk.com/about/disease.htm). Pentavalent antimonial or SSG, which has long been the first line drug, is no longer recommended for use as high levels of resistance in the Indian subcontinent have been reported (21). The other second line drugs like amphotericin B, its liposomal formulations and miltefosine are being used in the treatment with more efficacies and dramatic potential for curing leishmaniasis however, they are comparably costlier than the generic antimony (22). Other drugs like paromomycin and pentamidine have shown some usefulness and could be a potential supplement in the drugs regimen but their use and availability in disease endemic regions is limited (22-24). Identification and characterization
of cellular targets and answering the problem of drug resistance in leishmaniasis has always been the main thrust of protozoan research worldwide. The recent advancements in innovative animal models and parasites with reporter gene constructs have provided rapid and high through output drug screening methods in both, in vivo and in vitro (25-26). Several other drugs, in particular the antifungal azoles itraconazole, ketoconazole, and fluconazole, have been on limited clinical trials, but the results were equivocal. At the same time as these new therapies are becoming accessible for the treatment of leishmaniasis, the use of the standard pentavalent antimonial [Sb (V)] drugs for VL, such as sodium stibogluconate, is threatened by the development of drug resistance. In practice, yet, their extensive use in poor countries is hampered mainly owed to high costs and also owed to concerns of toxicity and emergence of resistance (9). In response to concerns about preserving the currently accessible antileishmanials, especially in regions with anthroponotic parasite transmission, there is growing interest on combination treatments. In addition, there is increasing awareness that drug treatment can be complicated by variation in the sensitivity of Leishmania species to drugs, difference in pharmacokinetics, and variation in drug-host immune response interaction. This article will focus on the factors that cause difference in response to antileishmanial chemotherapy, assess the tribulations connected by medical and obtained resistance, and deem how a method for screening and observation might be executed with connected insinuations for investigate, drug utilize, and communal fitness manage.

**Table 1. Recent accessible drugs for visceral leishmaniasis**

<table>
<thead>
<tr>
<th>Drugs position</th>
<th>Drugs</th>
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<tbody>
<tr>
<td>First-line drugs</td>
<td>Sodium stibogluconate (Pentostam); meglumine antimoniate (Glucantime)</td>
</tr>
<tr>
<td></td>
<td>Amphotericin B (Fungizone)</td>
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<td></td>
<td>Liposomal amphotericin B (AmBisome)</td>
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<tr>
<td></td>
<td>Pentamidine</td>
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<tr>
<td>Clinical trials</td>
<td>Miltefosine (oral, phase IV)</td>
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<tr>
<td></td>
<td>Paromomycin (phase III)</td>
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<td></td>
<td>Sitamaqine (oral, phase II)</td>
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<tr>
<td></td>
<td>Other amphotericin B formulations</td>
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**Disease progression and host immune response**

Leishmanias are mandatory intracellular protozoan parasites. The parasites remain inside their vectors as extracellular promastigotes (27). Following sandfly bite, neutrophils voyage in the neighborhood and detain the parasites, however the latter have the aptitude to escape and subsequently invade the macrophages of the skin, where they discriminate and imitate as amastigotes (28, 29). From there, parasites disseminate and invade further macrophages of the reticulo-endothelial system, and lastly infiltrate the bone marrow, liver, and spleen (27). Folks who acquire defensive immunity (skin test positive) without ever having visceral leishmaniasis have a sturdy type 1 CD4+ response to leishmania antigens. Antigen specific interferon-gamma and proliferation, as well as the aptitude to kill intracellular leishmania, are hallmarks of defensive immunity (30, 31). Because visceral leishmaniasis patients lack these responses to leishmania and other antigens, they habitually expire of secondary infections unless treated. In addition, augmented interleukin-10 secretion is characteristic of the disease (32-34). VL should be regarded as a state of long-term parasitism, as leishmanias are not eliminated completely but
rather remain in skin macrophages for lifetime, even after triumphant treatment in hosts with intact T-cell immune responses. In skin, leishmanias work as a reservoir for the prospective relapse of symptomatic VL. The jeopardy for relapse increases when T cell immune responses are impaired and irrespectively of prior antileishmanial treatment, as noted in HIV-infected patients (35–37). Relapses habitually peak 6–12 months after treatment.

Immunity to leishmaniasis is mediated by both arms of mammalian cellular immune system; innate (by neutrophils, macrophages, and dendritic cells) and adaptive (T cells) responses (38). The sand fly bite causes minimal tissue damage that promotes recruitment of neutrophils to the site of injury as a primary immune defense mechanism of the host (39, 40). Host protection against VL requires a pro-inflammatory, T helper (TH) 1 immune response, as characterized by the production of interleukin (IL)-12 by antigen presenting cells and IL-2, tumor necrosis factor alpha (TNF-α) and interferon (IFN)-γ by T cells (41, 42). Infected macrophages are activated by IFN-γ and TNF-α to kill intracellular amastigotes via the L-arginine nitric oxide pathway (43-45). Cured or subclinical patients are able to mount antigen-specific IFN-γ responses following Leishmania antigen stimulation in vitro. Treatment-cured individuals can be resistant to reinfection and become leishmanin skin test positive, suggesting no inherent defect in the Leishmania-dependent TH1 response (46-48). IL4 also plays an important role in effective antileishmanial chemotherapy, which appears to be modulated by IFN-γ- production (49). Deactivation of macrophages, suppression of Th1 responses, and dissemination of leishmanial infection are induced by IL10 (50). Increased IL10 levels have been detected repeatedly in human VL and are considered crucial in uncontrolled leishmanial infection (51, 50). Targeting IL10 has been associated with activation of Th1 responses and parasite killing, whereas IL10 suppression constituted a critical step in vaccine-mediated immunotherapy (52). Active disease in humans is associated with elevated IL-10 levels in serum and enhanced IL-10 mRNA in lesional tissues (33, 53). The presence of IL-10 is one factor that leads to a shift in the balance from a pro-inflammatory and effective immune response to a regulatory and dysfunctional immune response not capable of controlling disease progression. Another immunological parameter associated with disease progression and suppression of the immune response to VL is hypergammaglobulinemia (54).

Antimonials

Pentavalent antimonials, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), have remained the mainstream treatment for VL since their introduction in the 1940s and are still highly effective, except in regions in Bihar, India and Nepal, where resistance has rendered them almost useless (55, 2). A first course of antimonials, at the WHO-recommended regimen of 20 mg/kg of body weight/day for 28 to 30 days, led to clinical and/or parasitological response in 33 to 82% of European coinfeated patients, with relapses being common. The success rates in published studies vary greatly due to high levels of dropout and early death (56-60). However, the dose escalation strategy 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; these days, up to 60% of newly diagnosed VL cases in this area do not respond to antimonials (60). 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 (61, 62).
Low rates of antimonial resistance have been reported in Sudan also (63). 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 (64-67). 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 (65-67, 61). 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 (68-70). Recent evidence indicates that antimonials kill leishmanias by a process of apoptosis (70).

The reduction of pentavalent to trivalent compound takes place either in macrophages or in the parasite however, it is still a dilemma (71). Parasite mediated reduction has been found to be associate with the loss of reductase activity of parasite, which may also lead to drug resistance. This is supported by the observation that SbV resistant Leishmania donovani amastigotes lose their reductase activity. The recent finding of a parasite thiol dependent reductase (TDR) 1 enzyme, that catalyze the conversion of SbV to SbIII using glutathione as a reductant also supports this possibility (72). In addition, arsenate reductase 2 (ACR2) a new antimoniate reductase characterized in Leishmania sp. Increases sensitivity of parasite to SbV (73). It has also been reported that this reduction takes place primarily in macrophage rather than parasite (74). The supporting evidences that come from organisms like bacteria and yeast, where the metal reduction is mediated by host specific enzymes suggest that this conversion is host specific (75).

The routes of antimonials entrance into leishmania and macrophages are not well known. However, parasitic aquaglyceroporn, aquaporin 1 transporter is supposed to be responsible for the transport of antimonials into amastigotes (76). In addition, the transport of SbV, is able to enter the parasite (75). Both form of antimonials SbV and SbIII kills Leishmania species by DNA fragmentation, suggesting the role of apoptosis, β oxidation of fatty and adenosine diphosphate phosphorylation. However, the exact mechanisms of action are still unexpected (77-79). In addition, the antimonials inhibit glycolysis and metabolic pathways and increases efflux of intracellular thiols by an ATP binding cassette (ABC) transporter, multi drug resistant protein A (MRPA) (80). Pentamonials are also known to inhibit trypanothion reductases, an enzyme responsible for protection from host reactive oxygen and nitrogen species to parasites (81). The widespread misuse of drug, as it was easily accessible over the counters in endemic regions; along with loss of drug activation by parasites are the major causes of acquired resistance. The in vitro studies on SbV resistant Leishmania axenic amastigotes and promastigotes indicate their diminished ability to reduce SbV to SbIII (26). A study on amastigote and promastigotes forms of SbIII resistant leishmania, have shown reduction in accumulation of metals due to either reduced uptake or increased efflux (82). Overexpression of a heat shock protein (HSP70) gene has been found to be associate with the antimonial resistance (83). The transporters of ABC family, MRPA and pentamidine resistant protein 1 (PRP1) that act as efflux pump for antimonials, are also linked to antimony resistance (84, 85). Further, various genes identified in antimonial unresponsive clinical isolates suggests the multifactional mechanism of resistance (86-89).

**Amphotericin B**

Amphotericin B (AmB) is a polyene antifungal drug extensively used to treat systemic fungal infections (90). In widespread areas of Bihar, India where antimonials resistance in common,
AmB show high affinity for ergosterol, the predominant sterol of fungal and leishmanial cell membrane. Despite its high efficiency, AmB is also toxic and its side effect has been reported (91, 92). It 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 Bihar State, India VL cases. Unresponsiveness and relapses occur rarely, except among HIV-infected patients (68, 36, 37). In this population, secondary episodes of VL are common and are attributed mainly to relapse but also to reinfection (36). 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 (9). 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 (9). For immunocompetent patients, no treatment failures have been observed with the currently recommended total dose of 20 mg/kg (93), except in Sudan. Sudanese patients without HIV were less responsive to L-AmB than were Europeans or Indians: the cure rates for total doses of < 15 mg/kg, 16 to 20 mg/kg, and >20 mg/kg were 50%, 64%, and 88%, respectively (94). In another study, 10 of 64 Sudanese patients did not respond to treatment with L-AMB (20 mg/kg). This was thought to be related to high initial parasite loads and immunosuppressing underlying diseases (HIV infection and tuberculosis), and it was suggested that higher doses should have been used (95). In Europe, total doses of up to 30 to 40 mg/kg have been evaluated in small numbers of coinfected patients and were well tolerated, but they did not prevent relapses (96, 97). A dosing recommendation for coinfected patients cannot be made based on the limited data available.

Adverse effects of plain AmB have been circumvented with its three clinical formulations in which deoxycholate have been replaced by other lipids. Three formulations are liposomal AmB (L-AmB: Ambiosome), AmB colloidal dispersion (ABCD: Amphocil) and AmB lipid complex (ABL: Alelcit). These lipid formulations of AmB retain their antifungal activity and show very high efficacy to cure this deadly disease and less toxic. In VL cases, liposomal AmB has been proved as an efficient drug with more than 95% efficacy but high cost limits its use to common man suffering from this deadly disease. AmBisome is the only Food and Drug Administration approved lipid preparation for treatment of VL and has been most widely tested. Results from a recent three-arm study in India demonstrated that i) AmBisome and Abelcet (each given at a dose of 2 mg/kg/day for 5 days) produced far fewer infusion-related reactions versus conventional amphotericin B (15 alternate day 1 mg/kg infusions over a 30-day period) and little of the other toxicity of the latter drug (e.g., renal insufficiency, hypokalemia, anemia); ii) AmBisome induced significantly fewer infusion reactions and more prompt defervescence versus Abelcet; and iii) overall cure rates appeared similar (amphotericin B = 96%, AmBisome = 96%, Abelcet = 92%) (98). Findings in a tri-continental AmBisome study have suggested regional variations in clinical and parasitological responsiveness in patients with VL: total doses required for 100% cure were low in India (6 mg/kg, Leishmania donovani), higher in Kenya (14 mg/kg, L. donovani), and highest in Brazil (> 20 mg/kg, L. chagasi) (14). Similarly high total doses of AmBisome (18–20 mg/kg) are also needed in the Mediterranean region (L. infantum [identical to L. chagasi]) (99-101). 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 (9, 67). The use of nanoparticles and microspheres for the delivery of conventional amphotericin B also increased its efficacy against experimental VL (102-104). Similar results have been reported with the heat-induced reformulation of amphotericin B (105).
The antileishmanial activity of AmB and its lipid formulation is due to its interaction of both sterols i.e. ergosterol of leishmania and cholesterol of host macrophages. Since cholesterol is complexed by AmB, it markedly inhibits binding *leishmania donovani* promastigotes to macrophage (106). Further, at higher concentration (<0.1 M), it induces the formulation of aqueous pores in leishmanial promastigotes cell membrane that result in osmotic changes leading to cell lysis (107). Inspite of excellent efficacy the administration of AmB is also associated with the toxicity and emergence of parasitic resistance. The damaging effect of AmB in kidney tubular cell is mainly due to increased salt and Ca$^{2+}$ concentration, H$^+$ permeability across the aqueous pores that lead to sustained collapse of pH and Ca$^{2+}$ gradient across the membrane, a mechanism responsible for apoptosis in eukaryotic cells. The *in vitro* studies demonstrate that resistant leishmania lacks ergosterol, the main target of AmB (108). In *Leishmania donovani* AmB resistant strain, parasitic cell membrane lacks C-24 alkylated sterols that might be due to inactivation of enzyme S-adenosyl methionine transferase which is responsible for alkylation at C-24 position in ergosterol moieties leading to resistance (109). Another study has shown that resistance to AmB was found to be associated with gene TarII 64.4 and tarII 512.2 amplification in Leishmania tarentolae mutant cell lines (110). Till date clinical resistance against AmB is not reported but the relative nonspecific mode of action of AmB at the level of membrane may be a factor for its infrequent resistance. It has been shown in a study that success of AmB treatments greatly depends on patient’s immunity status and indicate that successive relapse could enhance emergence of AmB resistant isolates (111, 36). These finding warrants the possibility of resistance against the most successful drug.

**Miltefosine**
Miltefosine is initially developed as antineoplastic agent, which is an alkylphosphocholine (hexaadecylphosphocholine) moiety (12). It is the first oral drug used for the treatment of VL and was considered as major breakthrough in antileishmanial chemotherapy (112,113). Its phase I/II/III trials provoked a storm of protection against VL that was followed by phase IV trial, which also proved its relevance in outpatient setting in those areas where VL is endemic (114, 115). 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 (115). In this study, 3% of patients developed adverse effects, mainly gastrointestinal toxicity, and elevated hepatic transaminases and creatinine (115). 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, 50mg/kg/day in adults <50 kg, and 2.5mg/kg/day in children (maximum dose: 100 mg/day). Miltefosine has a half-life of ~1 week, so especially in relapsing coinfected patients, there is a risk of resistant parasites developing (116), as generated in vitro (117). In India, where large-scale miltefosine distribution is planned within the context of an elimination program, controlled use and knowledge of the HIV status of patients are of crucial importance (118). In Europe and other zones where VL transmission is zoonotic, the use of miltefosine (and other first-line drugs) should not be allowed in dogs to prevent the development of primary resistant strains (118). Even before miltefosine is introduced into the market or into control programs, preliminary data from a phase IV trial in India involving domiciliary treatment with miltefosine and weekly supervision suggests doubling of the relapse rate (119); this provides warning that drug resistance could develop quickly and plans are required to prevent it.
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 (120). It has been found that miltefosine induces an apoptosis like cell death in L. donovani, by producing numerous defects (120). 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 (37, 120). Recently, it was found that miltefosine enhanced IFN-γ receptors and thus IFN-γ responsiveness in L. Donavan-infected macrophages; in the same model, miltefosine induced an IL-12-dependent Th1 response and reversed the Th2 response to Th1 response (121). Resistance to miltefosine may emerge easily during treatment due to single point mutations (122, 117). 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 (120, 122). 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 (120). 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 (120, 122). Furthermore, modifications in lipid compositions of membranes and sterol biosynthesis have been detected in miltefosine-resistant L. donovani promastigotes (123). Since membrane fluidity and permeability are influenced by lipid composition, their modification may affect drug-membrane interactions (123). A case of a healthy patient with VL who relapsed 10 months after successful treatment with miltefosine for 28 days was reported recently (124).

**Pentamidine**

Pentamidine is an aromatic diamine used to cure leishmaniasis as a second line drug. Its isethionate and methansulphonate as a second line drug. Its isothionate and methansulphonate salts are mainly used for the treatment of VL. It was initially used to treat Sb-refractory patients in India but its declining efficacy and high resistance risk has led to its closure in India. Some combinational strategies have also been tried with this drug. A study on antimony unresponsive patient revealed that combination of low dosage of pentamidine are allopurinol as compare to the full dosages of pentamidine are more effective and less toxic with and ultimate cure of 73% and 58%, respectively (125). Pentamidine, although toxic in treatment doses, has been considered for use in secondary prophylaxis (126), as intermittent low-dose maintenance therapy is unlikely to show the toxicity seen with a therapeutic dosing regimen. Following a recent agreement between WHO and the manufacturer (Sanofi-Aventis), pentamidine is now available for free for the treatment of all neglected diseases, including leishmaniasis. Assessment of a diamidine compound (pentamidine isethionate) in the treatment-resistant cases of kala-azar is occurring in North Bihar, India (127, 128). The antileishmanial mechanisms of action of pentamidine, which possibly include inhibition of polyamine biosynthesis, DNA minor groove binding, and effect on mitochondrial inner membrane potential, are still not clearly defined (129). Pentamidine-resistant promastigote clones of L. donovani and L. amazonensis were shown to have 18- and 75-
fold reduced uptakes, respectively, and increased efflux (130). Although specific transporters for pentamidine uptake have been characterized and might have a role in resistance (129, 85), other data have also implicated the accumulation of pentamidine in the Leishmania mitochondrion as being of importance. Wild-type promastigotes accumulate more pentamidine in the mitochondrion in comparison to resistant cells. It is suggested that less organelle accumulation makes far more drug available for efflux (130). Although, its precise mode of action is not known, it is reported that the drug enters inside leishmaia donovani promastigote through arginine and polyamine transporters (131, 132). In a biochemical study it was found that in pentamidine resistant, leishmania donovani and leishmania amazonensis promastigotes clones, drug resistivity is due to decreased uptake followed by increased efflux of drugs. There is alteration in polyamine carrier that might be responsible for the alteration in surface protein nature and content leading to decreased influx of drug. Furthermore, this drug gets accumulated in mitochondria and enhances efficacy of mitochondria respiratoty chain complex II inhibitors suggesting its leishmanicidal activities due to decreased mitochondrial membrane potential. It is also reported that it inhibit mitochondrial topoisomerase II (133). It is also reported that it inhibit mitochondrial topoisomerase II (133). Pentamidine is highly toxic; causes hypoglycemia, nephrotoxicity and hypotension etc. Pentamidine resistance mechanism is not well understand, but intracellular ABC protein PRP1 can confer resistance to pentamidine in intracellular stage of leishmania (134).

**Paromomycin**

Paromomycin (Aminosidine) is an aminoglycoside-aminocyclitol antibiotic and has both antibacterial and antileishmanial activity. Paromomycin cures both, VL and CL (more effectively) but limited availability restricts its use in endemic regions (17, 135). In a phase III study of VL in India, this drug was associated with 94.6% cure rates, similar to amphotericin B (136). International efforts have successfully resurrected a newly manufactured preparation that is now being tested in India (once a day intramuscular injections of 15 mg/kg for 21 days). Assuming that its high-level efficay and low rate of adverse reactions are redocumented, the one drawback of paromomyacin (prolonged treatment duration) should be satisfactorily balanced by its proposed cost to be capped at US $45 (99). Since the 21-day schedule for this drug is also attractive compared with the 28−30 days for Sb or amphotericin B, paromomycin has the potential to replace amphotericin B (and residual Sb use) in India, and, if tested successfully elsewhere, could replace Sb in other regions as well. Paromomycin main side effects were a reversible elevation of liver enzymes, in approximately 1% of patients, and pain at the injection site (136). It is the cheapest of all antileishmanial drugs. Presently, paromomycin is under phase IV clinical trials (9). The mechanism of paromomycin action is largely unclear. Its *modus operandi* in Candida krusei supports cytochrome C inhibition but in Leishmania requires further elucidation. Recently it has been shown that cationic paromomycin binds to the negatively charged leishmanial glycocalyx suggesting mitochondria as a primary target (137). In addition, paromomycin inhibits translocation and recycling of ribosomal subunits and hence protein synthesis. Paromomycin in Leishmania donovani promotes association of 50S and 30S subunits of both, cytoplasmic and mitochondrial ribosomes and stop their recycling that eventually inhibits protein synthesis (138). Further exploration came from the study of Hirokoma et al that proves that paromomycin interacts with both 30S and 50S subunits without inhibiting the association of translation initiation factor-3 (IF3) to the 30S ribosomal subunit (139). Due to its limited use resistance is not yet reported in outpatient treatment but resistance has been reported
in vitro in leishmania donovani and leishmania tropica (137, 140). Since paromomycin is an aminoglycoside, it is possible that resistance will emerge rapidly if used as monotherapy.

**Azoles**

The biosynthetic pathway of ergosterol, the major sterol in fungi as well as Leishmania spp. and T. cruzi, is a target for the most important antifungal drugs. There has been an interest in two classes of these drugs as antileishmanial agents, the allylamines (for example, terbinafine) that inhibit squalene epoxidase and the azoles (for example, ketoconazole and itraconazole) that inhibit C14α-demethylase. A number of clinical studies have suggested that these sterol biosynthesis inhibitors are more effective against L. major and L. mexicana infections than against L. donovani or L. braziliensis infections. The only placebo controlled trial on the treatment of CL with ketoconazole showed that L. mexicana infections were more responsive than L. braziliensis infections (141). The results from in vitro studies that have investigated the intrinsic differences in sensitivity of Leishmania species to sterol biosynthesis inhibitors have not been in agreement. In a comparative study on the sensitivity of promastigotes to ketoconazole, L. donovani, L. braziliensis and L. amazonensis were found to be more sensitive than L. aethiopica, L. major, L. tropica and L. mexicana (142). These results contrast with those of Rangel et al. (143) who found that L. braziliensis was resistant to the activity of ketoconazole and the bis-triazole D0870 whereas L. mexicana was sensitive to ketoconazole. Both these results contrast with an earlier study, using an amastigote-macrophage model, which showed that L. donovani was more sensitive to ketoconazole than L. mexicana or L. major (144). Extensive studies in *Candida* spp. have shown that mutations at both the active site and heme cofactor site of cytochrome P450 sterol 14-demethylase (CYP51) can result in reduced sensitivity to azoles. Clinical resistance in *C. albicans* isolates has been shown to be due to drug efflux following upregulation of ABC and multidrug transporters as well as upregulation of several *ERG* genes that code for enzymes in the sterol biosynthesis pathway. There have been no published experimental studies on acquired resistance in *Leishmania* spp., but resistance to fluconazole was shown to be rapidly induced in vitro in the related parasite *Trypanosoma cruzi* (145).

**Sitamaquine**

Sitamaquine, a 4-methyl-6-methoxy-8-aminoquinoline (lepidine), previously known as WR6026, is in phase II trials for the treatment of VL. The drug has broad-spectrum antiprotozoal activity (146) but with limited clinical use and no reported resistance. It was originally developed in collaboration with GlaxoSmithKline and Walter Reed Army Institute (114). Sitamaquine, a primaquine analogue as an antimalarial drug has been reported to be toxic with low efficacy in kala-azar treatment (147, 148). Recent *in vitro* parasite evaluation confirmed the antileishmanial properties of sitamaquine dihydrochloride against a range of *Leishmania* species responsible for either cutaneous or visceral leishmaniasis, with ED50 values against amastigotes in a range from 2.9 to 19.0 microM (149). The advantage of this drug is its oral administration. In phase II assays in India with 120 VL patients (19), and in Kenya with 95 VL patients (20), sitamaquine was well tolerated with the doses ranging from 1.5 to 3 mg/mg/day. However, despite efficacy few side effects like vomiting, dyspepsia, cyanosis, nephritic syndrome and glomerulonephritis were also observed. The consequences of Kenyan phase II trial were different from India trial (20). The Kenyan trial showed somewhat equal efficacy but observed side effects were abdominal pain, headache and kidney dysfunctioning. Sitamaquine at high concentration affects parasite motility, morphology and growth (150). Mechanism of its action involves electrostatic interaction
between phospholipid anionic polar head groups and positively charged sitamaquine and then with phospholipid acyl chains leading to drug insertion within biological membranes (151). After binding to the membrane, sitamaquine accumulate in leishmania cytosolic acidic compartments, acidocalcisome. However, correlation between its action and accumulation is not clear (152). There is transient affinity between sitamaquine and membranes and also energy dependent efflux was demonstrated (151). Although, resistance against this drug has not been reported yet in clinical practices but, in vitro resistance against leishmania donovoni promastigote has been reported by selecting drug pressure of sitamaquine at 160 micro m concentration (153). In one of the study, conducted on cutaneous leishmaniasis caused by leishmania major on BALB/c mice, sitamaquine dihydrochloride did not reduce the parasite burden and lesion progression was continued. The lack of its efficacy and activity seriously restricted further clinical trials (149).

However, with available status of knowledge, more studies are required to understand its efficacy, mode of action as well as toxicity. The major advantages of sitamaquine are its administration route and pharmacokinetics characteristics. Thus, its bioavailability is better than those of miltefosine. It is now probable that GSK company, the developer, will take a decision concerning the marketing of sitamaquine in the next future.

Nucleoside analogues
Wide variations in sensitivity of promastigotes of different species to the pyrazolopyrimidines allopurinol and allopurinol riboside were reported suggesting differences in the affinity of enzymes of the purine salvage pathway (154, 155). In the 1980s, allopurinol, a pyrazolopyrimidine, entered clinical trials for the treatment of VL and CL, both alone and in combination with antimonials (13). Allopurinol is known to inhibit enzymes of the purine salvage pathway in Leishmania (154). In comparative studies wide variations in sensitivity of the promastigotes of different species to the pyrazolopyrimidines allopurinol and allopurinol riboside were reported to be due to differences in the affinity of enzymes of the purine salvage pathway (154, 155). The mode of action of allopurinol is thought to involve conversion to ribonucleoside triphosphate analogues and incorporation into RNA, thereby disrupting macromolecular biosynthesis (156). The pharmacokinetic properties are a major limitation to the use of allopurinol or its derivatives for treatment of human leishmaniasis.

Approaches to protect the efficacy of recently accessible antileishmanials
Examining drug resistance requires methods that are able to determine either (I) the sensitivity of parasite isolates, or (II) molecular changes that indicate alterations in either the drug target or mechanisms that alter the intraparasite level of active drug. Both approaches suffer from major limitations. Determining drug sensitivity of isolates taken from patients is always open to the criticism that removal of microorganisms from the host and adaptation to culture media immediately selects for a subpopulation of pathogens best suited for growth in that medium. The drug sensitivity of parasites should therefore be tested as soon as possible after isolation from the patient. Ease of culture, speed of test and ease of quantification would point to a promastigote assay as best to fulfill this role. However, a major drawback to this approach is that promastigotes are not sensitive to pentavalent antimonials or some other drugs, for example the plant product PX-6518 (L. Maes, this volume) and have reduced sensitivity to yet others, for example paromomycin. In contrast promastigotes are more sensitive to pentamidine than amastigotes in vitro (157). The amastigote-macrophage assay offers the only model able to correlate clinical response to the sensitivity of the isolate. This has been demonstrated in relation
to pentavalent antimonials by Ibrahim et al. (158) and Lira et al. (159) who showed the correlation with the amastigote assay but not with the promastigote assay.

In addition to inherent pharmacologic features, there is a number of human parameters that may favor the emergence and spread of leishmanial resistance. These comprise poor compliance, costly treatment, availability of antileishmanial drugs over the counter, and limited access to healthcare facilities for early diagnosis and treatment. Given the recent situation of the widespread emergence of antimonial resistance in India, there is growing anxiety to preserve the efficacy of novel antileishmanials. Such an approach should focus on the following axons. (i) Treatment of VL should be based on guidelines for punctual 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 an approach of treatment with antimonials (first option) or amphotericin B (second option), based on culture and susceptibility results, compared with an empiric treatment approach, 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 (160). (ii) In order to augment compliance, directly observed therapy for antileishmanials should be implemented, like in tuberculosis control programs. (iii) VL cases should be treated early in order to avoid further transmission of resistant parasites in the community. (iv) Circulation and clinical response of antileishmanials should be monitored. (v) Antileishmanial treatment should be provided free of charge through the health-care system. (vi) The emergence and spread of antileishmanial resistance should be monitored. (vii) The efficacy and safety of combination regimens should be evaluated in large trials.

Conclusions
The managed of Visceral Leishmaniasis worldwide is dared by the prevalent emergence of antimonial resistance in Bihar State, India. No molecular markers of resistance are available for presently used antileishmanial drugs. The only consistent method for monitoring resistance of isolates is the technically demanding in vitro amastigote-macrophage model. The last decade new formulations of predictable antileishmanial drugs as well as latest agents became accessible. The extensive use of the oral agent miltefosine was hampered by the prospective for teratogenicity and emergence of resistance. The last years several mechanisms of infield antileishmanial resistance were identified. Considerate their molecular and biochemical characteristics will guide the design of novel drugs and also the molecular observation of resistance. New treatments for visceral leishmaniasis have been introduced and others are on clinical trial. Care needs to be taken that resistance to these drugs does not develop and regimens of simultaneous or sequential combinations need to be considered as well as systems to monitor drug use, drug response, and spread of resistance. On the whole the improvement of antileishmanials has been usually sluggish; novel drugs are needed.

Acknowledgments
The financial supports received from Indian Council of Medical Research, Government of India, New Delhi (5/8-7 (93) / 2010-ECD-II) is greatly acknowledged.

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