

# Drug Resistance in Leishmaniasis

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## INTRODUCTION

Leishmaniasis is a disease complex caused by 17 different species of protozoan parasites belonging to the genus *Leish-*

*mania*. The parasites are transmitted between mammalian hosts by phlebotomine sandflies. There are an estimated 12 million humans infected, with an incidence of 0.5 million cases of the visceral form of the disease and 1.5 to 2.0 million cases of the cutaneous form of the disease. Leishmaniasis has a worldwide distribution with important foci of infection in Central and South America, southern Europe, North and East Africa, the Middle East, and the Indian subcontinent. Currently the main foci of visceral leishmaniasis (VL) are in Sudan

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TABLE 1. Current drugs for leishmaniasis (parenteral administration unless otherwise stated)

Type of leishmaniasis	Status of drugs	Drugs
Visceral	First-line drugs	Sodium stibogluconate (Pentostam); meglumine antimoniate (Glucantime) Amphotericin B (Fungizone) Liposomal amphotericin B (AmBisome) Pentamidine
	Clinical trials	Miltefosine (oral, phase IV) Paromomycin (phase III) Sitamaquine (oral, phase II) Other amphotericin B formulations
Cutaneous	First-line drugs	Sodium stibogluconate (Pentostam); meglumine antimoniate (Glucantime) Amphotericin B (Fungizone) Pentamidine Paromomycin (topical formulations with methylbenzethonium chloride or urea)
	Clinical trials	Miltefosine (oral, phase III; registered in Colombia in 2005) Paromomycin (topical formulation with gentamicin and surfactants, phase II) Imiquimod (topical immunomodulator, phase II) Antifungal azoles (ketoconazole, fluconazole, itraconazole)

and India and those of cutaneous leishmaniasis (CL) are in Afghanistan, Syria, and Brazil. In addition to the two major clinical forms of the disease, VL and CL, there are other cutaneous manifestations, including mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), recidivans leishmaniasis (LR), and post-kala-azar dermal leishmaniasis that are often linked to host immune status.

The number of cases of leishmaniasis is probably underestimated as leishmaniasis is a reportable disease in only 40 of the 88 countries where it is known to be present (<http://www.who.int/tdr/diseases/leish/diseaseinfo.htm>). Although the global burden of leishmaniasis has remained stable for several years, causing the loss of 2.4 million disability adjusted years, there are also changing patterns of disease. Increasing numbers of human immunodeficiency virus (HIV) coinfections, human migration, and resettlement, especially important where leishmaniasis is zoonotic, make resurgence a possibility (47). Improved approaches to diagnosis, vaccine development, vector and reservoir control, and new drugs for treatment are being addressed (43).

The current situation for the chemotherapy of leishmaniasis is more promising than it has been for several decades with both new drugs and new formulations of old drugs either recently approved or in clinical trial (Table 1) (37, 39). The chemical structures of the commonly used drugs are given in Fig. 1. 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, 105); oral miltefosine (142) which has been registered in India and is now in phase IV trial; a parenteral formulation of aminosidine (paromomycin) (150) currently completing phase III clinical trials in India ([www.iowh.org](http://www.iowh.org)) and on trial in East Africa ([www.dndi.org](http://www.dndi.org)); and oral sitamaquine (previously WR6026), which has completed phase II trials in India, Kenya, and Brazil (50, 84, 159) and is in development with GlaxoSmithKline (<http://science.gsk.com/about/disease.htm>). Treatment of CL has also improved through the introduction of topical formulations of paromomycin (8, 55, 138) and other drugs, including

the immunomodulator imiquimod (6, 67). Some forms of CL also respond to oral miltefosine and phase III trials have been reported in Central and South America (137). 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 available 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 addition, there is increasing awareness that drug treatment can be complicated by variation in the sensitivity of *Leishmania* species to drugs, variation in pharmacokinetics, and variation in drug-host immune response interaction. For CL, the absence of baseline data from controlled clinical trials of established drugs adds to the problem of interpretation and the definitions of treatment success and failure. This review will focus on the factors that cause variation in response to antileishmanial chemotherapy, evaluate the problems associated with clinical and acquired resistance, and consider how a system for monitoring and surveillance might be implemented with associated implications for research, drug use, and public health control.

## HOST FACTORS

### Host Immune Status

The immune status of leishmaniasis patients has long been known to affect drug efficacy. This has proven to be of particular importance in relation to pentavalent antimonial treatment of DCL (59) and coinfections with HIV in the visceral form (12, 48), where there is an absence of a specific T-cell-mediated immune response and mutual exacerbation of infection. The basis for this lack of activity of pentavalent antimonials has been explored in immunodeficient mouse models for which the effects are probably due to deficiencies of both Th1-cell-mediated and macrophage responses (109). Experimental models have shown that the antileishmanial activity of

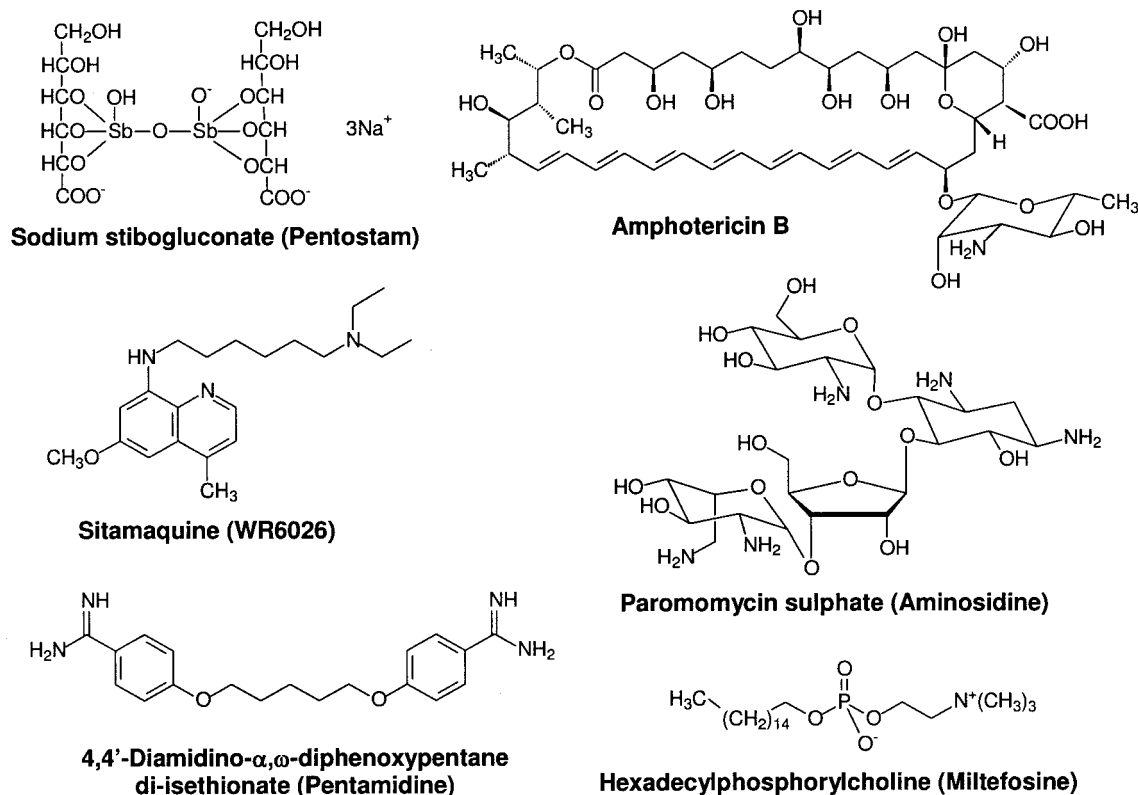


FIG. 1. Drugs currently used in the treatment of leishmaniasis.

pentamidine is also T-cell dependent whereas those of amphotericin B and miltefosine are T-cell independent (61, 108).

Irrespective of the findings of the experimental models, it is now known that intact immunity holds the key to the curative ability of antileishmanial drugs, including amphotericin B. Experiences with HIV/VL coinfection in the Mediterranean region, most frequently caused by *L. infantum*, suggest that CD4-deficient individuals tend to relapse frequently (90). In randomized controlled trials in Spain, cure rates in both antimonial- and amphotericin B-treated coinfecting patients were as low as 66% and 62%, respectively, compared with >90% cure rates in non-HIV patients (89). Similar figures have been reported in Ethiopia. In addition, >60% relapse of responders 12 months after completion of treatment was reported. In recent years there has been a decline in the incidence of VL in HIV-infected patients following the introduction of highly active antiretroviral therapy (98), again suggesting an important role for CD4 lymphocytes in preventing relapses and controlling the infection.

#### Pharmacokinetics

The pharmacokinetic properties of an antileishmanial drug can also determine efficacy as the sites of infection in leishmaniasis are in the visceral organs (bone marrow, liver and spleen), the skin or the nasal mucosa. To give two examples, sitamaquine (Fig. 1), an 8-aminoquinoline is well distributed to the liver (29) and is being considered for treatment of VL, whereas the antifungal itraconazole (a triazole) is well distrib-

uted to the skin (95) and has been on trial for the treatment of CL. Two other aspects of pharmacokinetics, metabolism and excretion, also require consideration. In a study on the treatment of CL in Saudi Arabia, patients showed marked variation in response to sodium stibogluconate (Pentostam) even though no differences were observed in the sensitivity of *Leishmania major* isolates to this drug from patients in the amastigote-macrophage model (3). However, significant differences were observed between patients in the elimination rate of antimonials and area under the curve analysis suggested that differences in the length of exposure to antimony could influence clinical response in CL treatment (4).

There have been few studies on the metabolism of established antileishmanial drugs. Experimental studies have suggested visceral infection itself might alter drug metabolism by decreasing cytochrome P450 levels in liver tissue (35, 127, 135). The mechanisms are not fully understood; availability of heme, alteration in protein synthesis, and/or degradation or inhibition by NO as a result of an immune response have been proposed. There have been no reported studies on variation of drug metabolism in VL or CL patients.

#### LEISHMANIA-RELATED FACTORS

There are over 17 species of *Leishmania* known to be infective to humans. The species have been characterized on the basis of biochemical and molecular differences and these differences provide a structure for phylogenetic analysis and improved methods of species identification and diagnosis (42,

94). Given the known biochemical and molecular differences between species it is perhaps unsurprising that there is variation in intrinsic sensitivity between *Leishmania* species to several drugs. Although such variation has been reported in laboratory studies, careful interpretation is of prime importance as different assay conditions can lead to severalfold differences in activity values (36). Despite this caveat, there is ample evidence of variation.

The second element of variation in response comes from selection due to drug pressure. The selection of drug-resistant pathogens is a major and well-known threat to the treatment of bacterial, viral, and fungal infections as well as some parasitic infections, such as malaria. Although resistance mechanisms differ between prokaryotic and eukaryotic organisms, some general principles can be identified (68, 79). The primary effect in cell killing is the interaction of a drug with one or more targets. Thus, the alteration of the intracellular drug level or the ability of the drug to affect the target is commonly observed in a wide variety of organisms. Drug levels at the target site of action can be lowered by a variety of mechanisms, including decreased uptake, increased export, and inactivation by metabolism or sequestration. Likewise, alterations in levels of primary target can occur due to decreased target affinity for the drug or complete loss of target, usually associated with a bypass mechanism.

Complex downstream events leading to cell damage and death are often triggered by inhibition of a primary target. For example, many antiparasitic drugs (e.g., nifurtimox, primaquine) undergo futile-redox cycling, producing reactive oxygen species that can peroxidatively damage membrane lipids, proteins or DNA (52, 66). Thus, overexpression of various repair systems can also play a role in drug resistance. Multiple mechanisms are frequently involved.

### Antimonials

**Species variation.** Variation in the clinical response to the pentavalent antimonials sodium stibogluconate, and meglumine antimonate (Glucantime) in VL, CL, and MCL has been a persistent problem in the treatment of leishmaniasis over the past 50 years. One explanation for this phenomenon is the intrinsic difference in species sensitivity to these drugs. In general, studies using the amastigote-macrophage model, *L. donovani* and *L. brasiliensis* were found to be three- to fivefold more sensitive to sodium stibogluconate than *L. major*, *L. tropica*, and *L. mexicana* (5, 13, 111). This was also shown in earlier studies by Berman et al., using another amastigote-macrophage model, which also demonstrated a wide variation in the sensitivity of isolates from cutaneous leishmaniasis cases to pentavalent antimonials (15). In one controlled clinical trial in Guatemala that compared the cure rate to antimonials of CL caused by different species (110), sodium stibogluconate produced a significantly higher cure rate in patients with *L. braziliensis* (96%) lesions than those with *L. mexicana* lesions (57%).

**Clinical resistance.** Pentavalent antimonial drugs were used worldwide for the treatment of VL and CL for over six decades with little evidence of resistance. Although the selection of resistant *Leishmania* has long been a part of laboratory studies, it is only in the past 15 years that acquired resistance has become a clinical threat. In most parts of the world, over 95% of previously untreated patients with VL respond to pentava-

lent antimonials, the recommended first-line treatment. However, the region endemic for VL in North Bihar, India, has the unique distinction of being the only region in the world where widespread primary failure to Sb(V) has been reported (141, 144, 154). Even in this geographical region a variation in Sb(V) sensitivity occurs with significant drug resistance at the epicenter of the epidemic and a high level of sensitivity only 200 miles away (144). This resistance is so far unique to *L. donovani*; all isolates from a large number of refractory as well as responding patients in India were identified as this species (146, 149).

Until the late 1970s, a small daily dose (10 mg/kg; 600 mg maximum) for short duration (6 to 10 day) was considered adequate, when unconfirmed reports suggested a 30% treatment failure with this regimen from four districts most severely affected, Muzaffarpur, Samastipur, Vaishali, and Sitamarhi (120) (see Fig. 3). Following this, an expert committee revised recommendations to use Sb(V) in two 10-day courses with an interval of 10 days and a significant improvement in cure rates (99%) was observed (2). However, only a few years later, another study noted 86% cure rates with 20 days of continuous treatment with this regimen (153). In 1984, a World Health Organization (WHO) Expert Committee recommended that Sb(V) should be used in doses of 20 mg/kg/day up to a maximum of 850 mg for 20 days, with a repeat of the same regimen for 20 days in cases of treatment failure. Four years later, Thakur et al. evaluated the WHO recommendations and reported that 20 days of treatment with 20 mg/kg/day (maximum 850 mg) cured only 81% of patients, although with an extension of the treatment for 40 days, 97% of patients could be cured (151). Three years later, the same group noted a further decline in cure rate to 71% after 20 days of treatment, and recommended extended duration of treatment in nonresponders (152). Jha et al. (83) found that extending the therapy until 30 days could cure only 64% of patients in a hyperendemic district of Bihar (Fig. 2).

From these findings it became clear that Sb(V) refractoriness was increasing although the reports came from studies that were not strictly controlled. In two following studies carried out under strictly supervised treatment schedules, it was observed that only about one-third of all VL patients could be cured with the currently prevailing regimen (144). The incidence of primary unresponsiveness was 52%, whereas 8% of patients relapsed. During the same period only 2% of patients from the neighboring state of (Eastern) Uttar Pradesh failed treatment (144). These studies confirmed that a high level of Sb(V) unresponsiveness exists in Bihar, though the drug continues to be effective in surrounding areas (Fig. 2). There are reports of antimony resistance spreading to the Terai regions of Nepal, especially from the district adjoining hyperendemic areas of Bihar, where up to 24% of patients seem to be unresponsive, though in eastern Nepal a 90% cure rate has been reported (124).

The reason for the emergence of resistance is widespread misuse of the drug. Sb(V) is freely available in India, and is easily accessible over the counter. Most patients (73%) first consult unqualified medical practitioners, who might not use the drug appropriately (147). It has been a common practice to start with a small dose and gradually increase the dose over a week. Drug-free intervals are given with the belief that they will prevent renal toxicity. On many occasions the daily dose



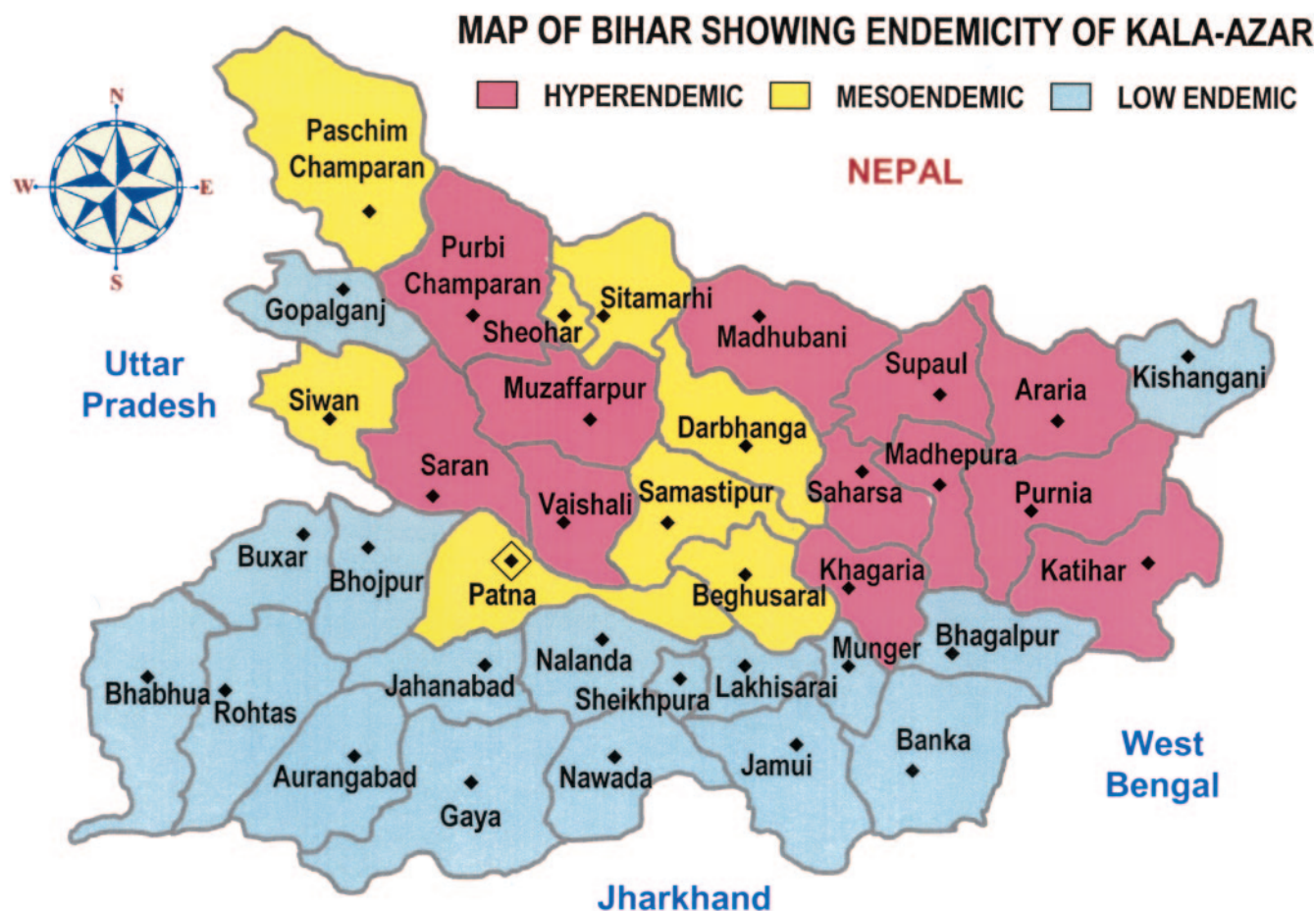


FIG. 2. Map of Bihar State, India, showing distribution of resistance to pentavalent antimonials in kala-azar-endemic areas.

of drug is split into two injections, to be given twice daily. These practices presumably expose the parasites to drug pressure, leading to progressive tolerance of the parasite to Sb(V). It has been observed that only a minority of patients (26%) were treated according to prescribed guidelines: irregular use and incomplete treatments were a common occurrence. These facts point to the mishandling of antileishmanial drugs in Bihar as a significant contributor to the development of drug resistance (147).

**Parasite resistance.** In a study to determine whether acquired drug resistance was present in Bihar, *L. donovani* isolates were taken from responders and nonresponders (96). Using an in vitro amastigote-macrophage assay, isolates from patients who did respond to sodium stibogluconate treatment were threefold more sensitive, with 50% effective doses ( $ED_{50}$ s) (around 2.5  $\mu$ g Sb/ml) compared to isolates from patients who did not respond ( $ED_{50}$ s around 7.5  $\mu$ g Sb/ml). There was no difference in the sensitivity of isolates when the promastigote assay was used (96). The significant difference in amastigote sensitivity supports the concept of acquired resistance in Bihar. However, more biological evidence is required to support the temporal and spatial parameters of the Bihar phenomenon. The sample size in this first study (96) was small

(15 nonresponders and 9 responders), and a threefold difference in sensitivity can be seen between experiments in this model (36).

Other reports on VL isolates from Sudan have also shown that the clinical response to sodium stibogluconate was reflected in isolates in the amastigote-macrophage model (but not in promastigotes) (1, 80). Other observations support the notion that Sb resistance can be acquired. In *L. infantum* isolates taken from immunodeficient and immunocompetent VL patients in France both before and after meglumine antimoniate treatment, isolates from 13 of 14 patients posttreatment had decreased sensitivity in an amastigote-macrophage assay (62). A similar decreased sensitivity was observed in *L. infantum* isolates taken from dogs before and after meglumine antimoniate treatment (74).

In the laboratory *L. donovani* resistance to antimonials is easily generated in culture, most recently in axenic amastigote of *L. donovani* and *L. infantum*, and a rodent model (58, 75). Although the in vitro data suggest that increasing the dose of Sb(V) could overcome the unresponsiveness, even the current doses produce unacceptable toxicity and further increase in the quantity of drug could seriously jeopardize the safety of the patients (146). What we still do not have is a marker of clinical

antimony resistance in *L. donovani* isolates. Several laboratory-generated markers of Sb resistance have now been identified (146), but evidence of their existence in field isolates from refractory patients has yet to be found. Although an amplicon was observed in a few isolates from Sb-refractory patients, the significance of this observation has yet to be determined (134).

The development of Sb resistance in the anthroponotic cycle in Bihar suggests that resistance could also develop to other antileishmanial drugs as they are introduced. A similar potential for resistance to develop exists in East Africa, especially in Sudan, another anthroponotic focus of VL with intense transmission, where poverty, illiteracy, and poor health care facilities portend misuse of the drug and consequent emergence of resistance. Resistance seems to be a feature of intensive transmission of anthroponotic *L. donovani* as epidemic turns to endemic in foci where Sb(V) has been used as monotherapy for long periods, often with poor supervision and compliance (146, 147). In other parts of the world, Sb(V) continues to be effective (34, 156). Another concern is that increasing numbers of HIV/VL-coinfected patients will be a potential source for emergence of drug resistance. These patients have high parasite burden and a weak immune response, respond slowly to treatment, have a high relapse rate, and could be a reservoir of drug-resistant parasites. Furthermore, the reports of transmission of infection via needle sharing in HIV/VL-coinfected patients in southern Europe, identify another route for spread of resistant parasites (40, 106).

**Mechanisms of action and resistance.** After 60 years of use, the antileishmanial mechanism of action of pentavalent antimonials is only now nearly understood. Interpretation of some of the earlier reports on mode of action and drug sensitivity to antimonials is complicated by the fact that liquid formulations of sodium stibogluconate contain the preservative *m*-chlorocresol, itself a potent antileishmanial agent (126). Unfortunately, much of this literature does not specify whether the liquid form or additive-free powder form was used. Nonetheless, it is now generally accepted that all pentavalent antimonials are prodrugs that require biological reduction to the trivalent form [Sb(III)] for antileishmanial activity. The site (amastigote or macrophage) and mechanism of reduction (enzymatic or nonenzymatic) remain controversial. However, several studies have reported that axenic amastigotes (i.e., cultured in the absence of macrophages) are susceptible to Sb(V), whereas promastigotes are not, suggesting that some stage-specific reduction occurs in this life cycle stage (27, 57, 58, 72). However, there are reports to the contrary (129). Certainly, a proportion of Sb(V) may be converted to Sb(III) in humans (25, 70) and in animal models (99), so both mechanisms may be operative. Further studies are required to resolve this issue.

Although stage-specific reduction has been demonstrated recently (132), the mechanism by which amastigotes reduce Sb(V) is not clear. Both glutathione and trypanothione can nonenzymatically reduce Sb(V) to Sb(III), particularly under acidic conditions (63, 65, 115, 162, 163). However, the physiological relevance of these observations is open to question since the rates of reduction are rather slow. Moreover, promastigotes contain higher intracellular concentrations of trypanothione and glutathione than amastigotes (7, 161) and both stages maintain intracellular pH values close to neutral, independent of external pH (69). Thus, it is difficult to account

for the selective action of Sb(V) against the amastigote stage by a nonenzymatic mechanism. As both stages can take up Sb(III) and Sb(V) the insensitivity of promastigotes to Sb(V) cannot be attributed to drug exclusion (22).

Two possible candidates for the enzymatic reduction of Sb(V) to Sb(III) in amastigotes have recently been identified. The first is a thiol-dependent reductase related to glutathione *S*-transferases that is more highly expressed in amastigotes (46). The second is a homologue of a glutaredoxin-dependent yeast arsenate reductase (167). The levels of expression of this protein in promastigotes and amastigotes were not reported and the low specific activity of the recombinant enzyme with glutaredoxin raises questions as to the physiological nature of the electron donor in *Leishmania* spp. The importance of these candidate proteins in conferring sensitivity to Sb(V) in amastigotes needs to be addressed.

There have been comparatively few studies on the mode of action of these drugs. Initial studies suggested that sodium stibogluconate [Sb(V)] inhibits macromolecular biosynthesis in amastigotes (18), possibly via perturbation of energy metabolism due to inhibition of glycolysis and fatty acid  $\beta$ -oxidation (16). However, the specific targets in these pathways have not been identified. More recent studies have reported apoptosis in Sb(III)-treated amastigotes involving DNA fragmentation and externalization of phosphatidylserine on the outer surface of the plasma membrane (130, 139). However, these effects do not involve the classical caspase-mediated pathway (130) and do not meet the more recent stringent definition of apoptosis (85).

The mode of action of antimony in drug-sensitive *L. donovani* involves several effects on glutathione and trypanothione metabolism (Fig. 3) (161). Exposure to Sb(III) causes a rapid disappearance of trypanothione and glutathione from isolated amastigotes and promastigotes in vitro. A significant portion of these thiols are effluxed from cells in approximately equimolar amounts with the remainder being converted intracellularly to their respective disulfides (trypanothione and glutathione). The formation of the latter was ascribed to continuing oxidative metabolism in the face of inhibition of trypanothione reductase. Sb(III), but not Sb(V), has previously been shown to be a time-dependent reversible inhibitor of trypanothione reductase in vitro (41). Since Sb(III) also inhibits recovery of intracellular thiols following oxidation with diamide, this is consistent with inhibition of trypanothione reductase in intact cells (161). The profound loss of these thiols (>90% in 4 h) coupled with the accumulation of disulfide (up to 50% of the residual within 4 h) causes a marked decrease in cellular thiol redox potential. Similar effects on thiol levels and thiol redox potential were observed when amastigotes were exposed to Sb(V), intrinsically linking the effects of the biologically active Sb(III) with the clinically prescribed Sb(V).

The mechanism by which *Leishmania* spp. acquire resistance to antimonials has been the subject of intensive research for several decades, often yielding apparently contradictory results. It should be borne in mind when evaluating the literature that (i) *L. tarentolae* is quite different to species that infect mammals, and (ii) some laboratory-derived promastigote resistant lines were initially generated by selection for resistance to arsenite (115) and subsequently found to be cross-resistant to Sb(III), whereas others have been directly selected for re-



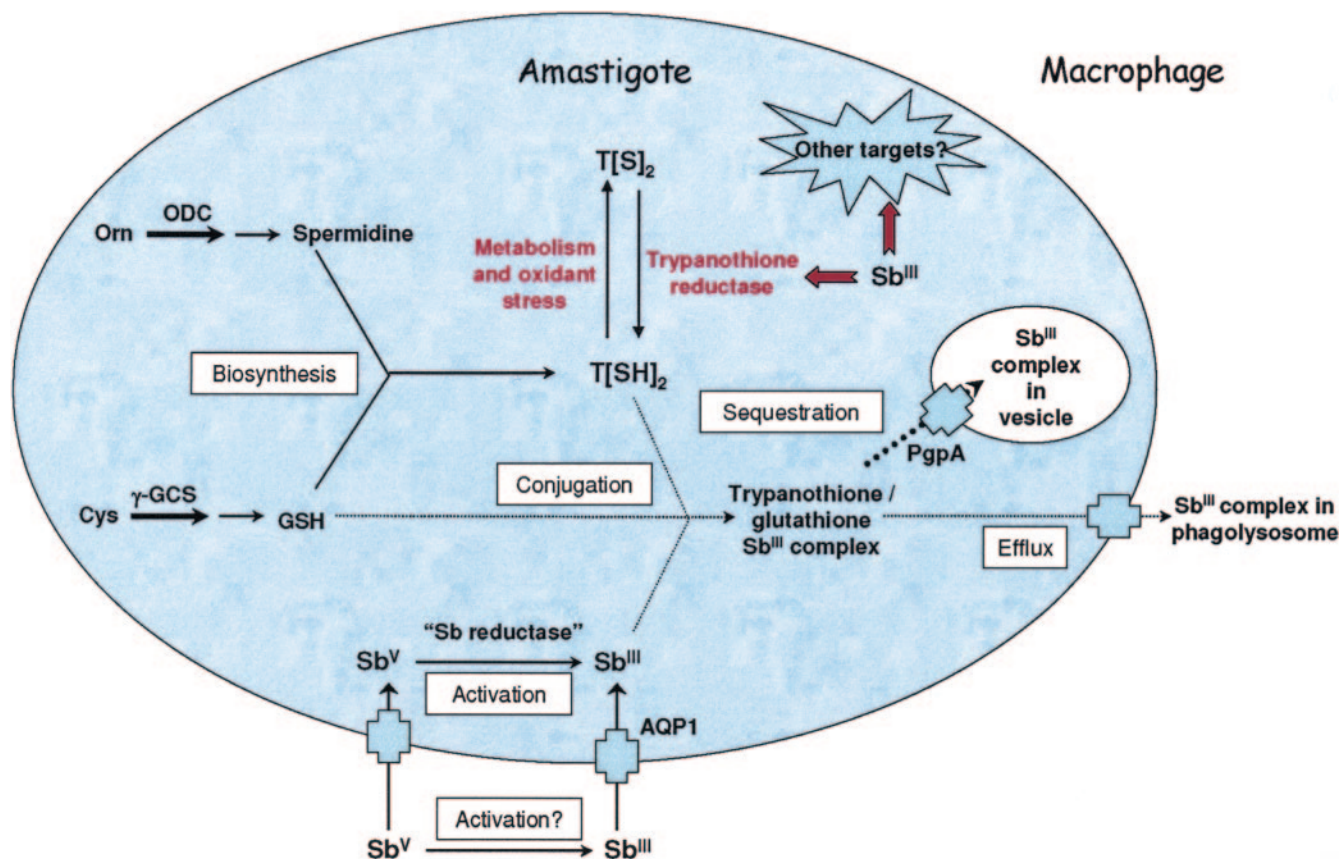


FIG. 3. Proposed mechanisms of antimony action and resistance in *Leishmania* spp. Levels of ornithine decarboxylase (ODC),  $\gamma$ -glutamylcysteine synthetase (GCS), and an intracellular P-glycoprotein (PgpA) are elevated in some laboratory-derived resistant lines (thick lines), whereas decreased Sb reductase is observed in others. Dotted lines indicate nonenzymatic steps implicated in resistance. The red arrow indicates inhibition of trypanothione reductase and other targets. Uptake of Sb(III) is mediated via an aquaglycoporin (AQP1).

sistance by exposure to Sb(III). While Sb and As are both metalloids, the selection method may affect the resulting resistance mechanism. As promastigotes are not sensitive to Sb(V), lines that were reportedly selected for resistance with Sb(V) preparations may have been selected for resistance to the *m*-chlorocresol preservative instead (58, 126). Alternatively, Sb(V) preparations could be partially reduced due to prolonged storage at acidic pH or in culture media containing thiols such as cysteine or glutathione (63, 65). It is also not inconceivable that some *Leishmania* spp. constitutively express higher amounts of "antimony reductase" activity in the promastigote stage than others.

Diminished biological reduction of Sb(V) to Sb(III) has been demonstrated in *L. donovani* amastigotes resistant to sodium stibogluconate (132). This line also shows cross-resistance to other Sb(V) drugs, but the same susceptibility to Sb(III) as the wild type (57), distinguishing it from the trypanothione pathway mutants described below. It is not known whether this mechanism occurs in clinical isolates at present. The accumulation of Sb(V) and Sb(III) in promastigotes and amastigotes has been shown to be by different transport systems (22), and although Sb accumulation was lower in resistant forms than in sensitive forms, levels of accumulation could not be correlated

to sensitivity in wild-type cells. Aquaglycoporins have recently been demonstrated to mediate uptake of Sb(III) in *Leishmania* spp. and overexpression of aquaglycoporin 1 renders them hypersensitive to Sb(III) (71). Transfection of aquaglycoporin 1 in an Sb(V)-resistant field isolate also sensitized it to sodium stibogluconate when in the amastigote form in a macrophage.

Increased levels of trypanothione have been observed in some lines selected for resistance to Sb(III) or arsenite (107). This is due to increased levels of the rate-limiting enzymes involved in the synthesis of glutathione ( $\gamma$ -glutamylcysteine synthetase) (76) and polyamines (ornithine decarboxylase) (78), the two precursor metabolites to trypanothione (Fig. 3). Increased synthesis of glutathione and trypanothione from cysteine could help to replace thiols lost due to efflux as well as to restore thiol redox potential perturbed by accumulation of disulfides (161).

Spontaneous formation of Sb(III) complexed with either glutathione, trypanothione or both has been demonstrated by proton nuclear magnetic resonance spectroscopy (140, 162) and by mass spectrometry (107). Since glutathione *S*-transferase (GST) is elevated in mammalian cells selected for resistance to arsenite (97), it has been proposed that formation of the metalloid-thiol pump substrates in *Leishmania* spp.

could be rate-limiting and that GST could mediate this activity (107). However, GST is not detectable in *Leishmania* spp., although there is an unusual trypanothione *S*-transferase activity associated with the eukaryotic elongation factor 1B complex (157).

The precise nature of the Sb-thiol complex remains uncertain, but two routes of elimination of the complex can be envisaged. The first involves sequestration in an intracellular compartment or direct efflux across the plasma membrane. Early studies noted that PgpA, a member of the ATP-binding cassette (ABC) transporters, is amplified in some resistant lines (26, 114). However, it soon became apparent that this transporter is not responsible for drug efflux across the plasma membrane. First, overexpression of PgpA was reported to decrease influx of Sb rather than increase efflux, possibly due to a dominant-negative effect through interactions with other membrane proteins (28). Second, overexpression of PgpA did not mediate increased efflux of radioactive arsenite from cells (49) or transport of arsenite across plasma membrane preparations (107). Finally, PgpA plays a relatively minor role in resistance (116) and is localized in membranes that are close to the flagellar pocket, the site of endocytosis and exocytosis in this parasite (91). Thus, the identity of the efflux pump in the plasma membrane and its role in resistance to antimonials remain to be determined. However, the studies described above have identified PgpA as functioning to sequester Sb(III) in an intracellular vacuolar compartment in *Leishmania* (Fig. 3). It is worth noting that resistance due to intracellular sequestration of Sb(III) as a thiol conjugate would show higher rather than lower intracellular levels of Sb(III). Thus, either sequestration plays a minor role in resistance or the conjugates must be rapidly exocytosed from the cell.

The next important step is to relate mechanisms observed in laboratory studies to clinical resistance. In one study on field isolates, no amplification of the genes found in laboratory studies was observed; rather amplification of a gene on chromosome 9 possibly involved in protein phosphorylation was identified (136).

### Amphotericin B

**Species variation.** Amphotericin B is a polyene antibiotic that has been used as a second line treatment for leishmaniasis since the 1960s. This compound (Fig. 1) has selective activity against fungi as well as *Leishmania* and *Trypanosoma cruzi*. Selectivity is due to the higher affinity of amphotericin B for ergosterol, the predominant sterol in these microbes, over cholesterol, the predominant sterol in the mammalian host cells. Differences in species sensitivity might be expected due to variation in the type and quantity of sterols in membranes of different species (11), a pattern more thoroughly characterized in relation to ergosterol biosynthesis in fungi (166). In a recent in vitro study on amastigotes of six species in the murine macrophage model, *L. mexicana* amastigotes were the least sensitive to this antibiotic (60). Amphotericin B in delivery systems might not follow the same pattern; the higher efficacy of liposomal amphotericin B against *L. donovani* than *L. infantum*/*L. chagasi* infections (14) is probably related more to parasite load and host immune status pathology than species sensitivity.

**Clinical resistance.** Although this antibiotic has been widely used in the treatment of mycoses for over 30 years, resistance in fungal isolates has been reported only rarely and this resistance was species dependent (54). There have been two small inconclusive studies on the emergence of amphotericin B resistance in *L. infantum*/HIV-infected cases in France. One study failed to find a change in sensitivity in promastigotes derived from isolates taken before and after the treatment of one patient (53). In contrast, a decrease in sensitivity was observed in isolates taken over several relapses from another patient (51). There has been increased use of amphotericin B for visceral leishmaniasis, in both the deoxycholate (142, 154) and lipid formulations (14, 143), following failure of antimonial treatment and in HIV/VL coinfection cases. With the increasing use of amphotericin B in lipid formulations that have longer half-lives, the possibility of resistance cannot be ignored.

**Mechanisms of resistance.** A resistant clone of *L. donovani* promastigotes was selected through a stepwise increase in amphotericin B concentration in culture. Resistant promastigotes showed a significant change in plasma membrane sterol profile by gas chromatography-mass spectrometry, ergosterol being replaced by a precursor, cholesta-5,7,24-trien-3 $\beta$ -ol (104). This probably results from a defect in C-24 transmethylation due to loss of function of *S*-adenosyl-L-methionine-C<sub>24</sub>- $\Delta$ -sterol-methyltransferase (SCMT). In *L. donovani* promastigotes two transcripts of the enzyme have now been characterized, one of which was absent in the amphotericin B-resistant clone, the other overexpressed but without a splice leader sequence which would prevent translation (121). This mechanism is different from some mechanisms of resistance to amphotericin B found in some fungi, for example *Cryptococcus* spp., for which defects in other isomerase and desaturase enzymes in ergosterol biosynthesis were shown (86). However, SCMT has been shown to be involved in reduced sensitivity of some *Candida* species to this drug (166). In the only other study on *Leishmania*, in this case the lizard parasite *L. tarentolae*, DNA amplification was observed with two extrachromosomal circles (134). All these studies have been performed with promastigotes and their importance in the intracellular amastigote has yet to be demonstrated.

### Miltefosine

**Species variation.** Miltefosine (hexadecylphosphocholine) has been recently introduced into the armory of antileishmanial drugs (37). The activity of miltefosine was compared to that of another phospholipid analogue, also initially developed as an anticancer drug, edelfosine (ET-18-OCH<sub>3</sub>). Variation in the sensitivities of both promastigote and amastigote stages of *L. donovani*, *L. major*, *L. tropica*, *L. aethiopica*, *L. mexicana*, and *L. panamensis* were shown in vitro (60). In all assays *L. donovani* was the most sensitive species, with ED<sub>50</sub>s in the range of 0.12 to 1.32  $\mu$ M against promastigotes and 1.2 to 4.6  $\mu$ M against amastigotes. *L. major* was the least sensitive species in the majority of assays, with ED<sub>50</sub>s for miltefosine in the range of 4.8 to 13.1  $\mu$ M against promastigotes and for miltefosine and edelfosine in the range of 7.5 to 37.1  $\mu$ M against amastigotes. *L. tarentolae* promastigotes have been reported to be 10-fold less sensitive to miltefosine than these *Leishmania*



species (118). More recently, studies on clinical isolates using a murine macrophage-amastigote model have confirmed the high sensitivity of *L. donovani* from both Sb-sensitive and Sb-resistant patients from Nepal (164). In the same study a significant lack of sensitivity of *L. braziliensis* and *L. guyanensis* isolates from patients in Peru up to 30  $\mu$ M was shown, in contrast to the sensitivity of *L. lainsoni* isolates (164).

**Clinical resistance.** The relevance of the above in vitro studies for clinical trials of miltefosine for the treatment of CL in Central and South America was indicated in reports published in 2004. In Colombia, where *L. panamensis* is common, the cure rate was 91% (38% for the placebo group), whereas in Guatemala, where *L. braziliensis* and *L. mexicana* are common, the cure rate was 53% (21% for the placebo group) (137). Isolates from responders and nonresponders were not taxonomically identified in this study; species identification needs to be given more priority in future studies in the region.

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 (145); this provides warning that drug resistance could develop quickly and plans are required to prevent it.

**Mechanisms of resistance.** Promastigote clones of *L. donovani* resistant to hexadecylphosphocholine up to 40  $\mu$ M have been generated in the laboratory. Resistance was stable after withdrawal of drug pressure. The lines showed no cross-resistance to standard antileishmanial drugs; the only cross-resistance was to the alkylglycerophosphocholine edelfosine (128). The mechanism of resistance in the 40  $\mu$ M miltefosine-resistant promastigote line was determined to be due to a >95% reduced accumulation of  $^{14}$ C-labeled miltefosine. However, binding of both drugs to the promastigote membrane and drug efflux was shown to be similar in sensitive and resistant lines (117). Subsequent studies by the Granada group identified a novel plasma membrane P-type transporter (LDMT gene) from the aminophospholipid translocase subfamily to be responsible for the uptake of both miltefosine and glycerophospholipids into *L. donovani* promastigotes. Two alleles with single distinct point mutations on this transporter were shown to be responsible for the reduced uptake (118). The potential relevance of these observations needs to be extended to miltefosine-resistant amastigotes before clinical implications can be properly considered.

Previously it had been shown that multidrug-resistant *L. tropica* lines that overexpress a P-glycoprotein are less sensitive to miltefosine (119). In contrast, P-glycoprotein overexpression was not observed in the 40  $\mu$ M-miltefosine-resistant promastigotes (128).

### Pentamidine

Pentamidine has been used as a second-line treatment for VL, CL, and DCL for over 40 years. Although use of pentamidine for the treatment of CL was revisited in the 1990s, with clinical trials for treatment of New World CL, this drug is not a widely used antileishmanial drug. This limited use, which is often in a zoonotic setting, suggests that development of resistance in CL species should not be a problem. However, for VL some indication that resistance could develop was reported.

During the short period pentamidine was used in India as a second-line drug for Sb(V)-refractory patients, there was a quick decline in the response rate from >95% cure rate in the early 1980s to <70% a decade later (81, 82). Evaluation of a diamidine compound (pentamidine isethionate) in the treatment-resistant cases of kala-azar is occurring in North Bihar, India (81, 82).

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 (21). Pentamidine-resistant promastigote clones of *L. donovani* and *L. amazonensis* were shown to have 18- and 75-fold reduced uptakes, respectively, and increased efflux (10). Although specific transporters for pentamidine uptake have been characterized and might have a role in resistance (21, 33), 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 (10).

### Paromomycin (Aminosidine)

**Species variation.** Paromomycin, an aminoglycoside-aminocyclitol antibiotic, has been used for the treatment of VL in a parenteral formulation in phase III clinical trials and CL in both topical and parenteral formulations. Some variation in sensitivity has been observed. In both experimental models and clinical cases of CL, lesions caused by *L. major* treated with paromomycin ointment resolved faster and more completely than lesions caused by *L. amazonensis* and *L. panamensis* (56). A more in depth in vitro analysis on the sensitivity of amastigotes in a murine macrophage model showed that *L. major* and *L. tropica* isolates ( $ED_{50}$ s in the range of 1 to 5  $\mu$ M) were more sensitive than *L. braziliensis* ( $ED_{50}$ , <12  $\mu$ M) and *L. mexicana* ( $ED_{50}$ , 39  $\mu$ M) isolates. *L. donovani* showed intermediate sensitivity ( $ED_{50}$ , 6 to 18  $\mu$ M), except for one Indian strain, DD8, which had an  $ED_{50}$  of >150  $\mu$ M (111).

**Clinical resistance.** Resistance to aminoglycosides in bacteria is well known and has been characterized in relation to decreased uptake in gram-negative pathogens, alteration of the ribosomal binding, and modification of amino groups or hydroxyl groups by inactivating *N*-acetyltransferases, *O*-phosphotransferases, or *O*-nucleotidyl transferases (44). Paromomycin has had limited use in the treatment of visceral leishmaniasis, and it is not surprising that cases of clinical resistance in this form of the disease have not been reported. However, it has been used more extensively for the treatment of cutaneous disease. So far there has been only one report suggesting resistance could develop. Following a 60-day parenteral course for treatment of two *L. aethiopica* cases, isolates taken from relapse patients were three- to fivefold less sensitive to the drug after treatment than isolates taken before treatment in an amastigote-macrophage assay (148). Monitoring of resistance could be of importance if paromomycin formulations are introduced as a first line treatment.

**Mechanisms of resistance.** The mechanisms of resistance in bacteria to this class of antibiotic are well characterized (see above). In bacteria aminoglycosides inhibit protein synthesis

through high-affinity docking to the 16S rRNA in the 30S ribosome subunit. There has been no similar description of the mechanisms of action of paromomycin in *Leishmania* spp. Mitochondrial ribosomes and induction of respiratory dysfunction and mitochondrial membrane depolarization have been implicated (101, 102).

In studies on selected populations of promastigotes, resistance was related to decreased drug uptake in *L. donovani* (100) but due neither to enzymatic modifications nor to any mutation of the small-subunit rRNA gene in *L. tropica* (64). In this study, analysis of small-subunit rRNA and DNA of a paromomycin-resistant *L. tropica*, which was eightfold more resistant to paromomycin and had a low level of cross-resistance to other aminoglycosides, showed no change in the sequence of the binding site. With the forthcoming introduction of paromomycin, there clearly need to be further studies to define the mechanisms of action and resistance in *Leishmania*.

### Azoles

**Species variation.** The biosynthetic pathway of ergosterol, the major sterol in fungi as well as *Leishmania* spp. and *Trypanosoma cruzi*, is a target for some of the most important antifungal drugs. Two classes of these drugs, the allylamines (for example, terbinafine) that inhibit squalene epoxidase and the azoles (for example, ketoconazole and itraconazole) that inhibit C14 $\alpha$ -demethylase, have generated the most interest as antileishmanials. The results from in vitro studies that have investigated the intrinsic differences in sensitivity of *Leishmania* species to sterol biosynthesis inhibitors have produced contradictory data. 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* (11). However, in contrast, Rangel et al. (122) observed that *L. braziliensis* was relatively insensitive to ketoconazole and the bistriazole D087, whereas *L. mexicana* was sensitive to ketoconazole. Both sets of results differ from those of an earlier study using an amastigote-macrophage model, which showed that *L. donovani* was more sensitive to ketoconazole than *L. mexicana* or *L. major* (13). The lack of concordance is probably due to different assay conditions, already shown to greatly influence antifungal activities, as well as the ability of amastigotes to salvage sterols, such as cholesterol, from host cell macrophages. This factor can reduce the sensitivity of this life cycle stage to azoles (125).

**Clinical resistance.** 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. One placebo-controlled trial on the treatment of CL showed that *L. mexicana* infections (89%) were more responsive than *L. braziliensis* infections (30%) to ketoconazole (110).

**Mechanisms of resistance.** 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* (24).

### 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 (165) but with limited clinical use and no reported resistance. In addition, there are no published comparative studies on *Leishmania* species sensitivity. Earlier studies reported similar ED<sub>50</sub>s for amastigotes of two species, 2.6  $\mu$ M against *L. tropica* (17) and 1.5  $\mu$ M against *L. donovani* (112). Sitamaquine was found to be 200 times more active than primaquine against *L. donovani* in hamsters in vivo but only twice as active as primaquine in vitro (87). Like primaquine, this compound appears to undergo hydroxylation and N-alkylation by rat hepatic microsomes (155). The activity of sitamaquine metabolites against *Leishmania* spp. has not been reported. The mode of action is not known but could involve "futile redox cycling" as proposed for primaquine.

### Nucleoside Analogues

In the 1980s, allopurinol, a pyrazolopyrimidine, entered clinical trials for the treatment of VL and CL, both alone and in combination with antimonials (39). Although not a successful treatment for human disease it is still used in treatment of canine leishmaniasis (88). Allopurinol is known to inhibit enzymes of the purine salvage pathway in *Leishmania* (113). 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 (9, 113). The mode of action of allopurinol is thought to involve conversion to ribonucleoside triphosphate analogues and incorporation into RNA, thereby disrupting macromolecular biosynthesis (103). The pharmacokinetic properties are a major limitation to the use of allopurinol or its derivatives for treatment of human leishmaniasis. Clinical pharmacology studies on the derivative allopurinol riboside, further to inefficacy determined following oral administration in clinical trials for treatment of CL in Central America, showed that, in contrast to plasma levels in dogs, plasma levels in humans were low, and there was incomplete absorption and metabolism by enteric gut flora (133).

### POLICY FOR LEISHMANIASIS AND DRUG RESISTANCE

In a 2001 review, Bryceson (23) stated "At the moment, there seems to be no policy at an international or national level to prevent the emergence of parasite resistance to antileishmanial drugs." Given the situation outlined above and the tools currently available, it is time that the measures and the policy to prevent the spread of drug resistance, as well as the development of resistance to new antileishmanials, be defined and implemented.

### Factors Involved in the Spread of Resistance

Two important considerations in an analysis of the importance of a drug resistance problem are the ease with which resistant individual microbes can be selected by a particular drug, and the potential spread of resistance in a population and, hence, the importance to public health. First, the spread of drug-resistant genotypes through a population of microorganisms is primarily governed by certain measurable parameters: (i) the volume (dose and frequency) of drug used, (ii) the probability that a drug-sensitive infection becomes resistant upon infection, (iii) the duration of infection in individuals, (iv) the fitness costs (division rate and transmissibility) for the pathogen incurred by being resistant in the absence of drugs, and (v) the degree to which compensatory mechanisms develop that offset these fitness costs (19, 45, 92, 93).

In zoonotic diseases, such as most cases of cutaneous leishmaniasis and most *L. infantum*/*L. chagasi* visceral leishmaniasis, the parasite is primarily an infection of a feral or domestic mammalian host and only occasionally infects humans. In zoonoses, the time that a parasite population is exposed to a drug is insignificant unless the mammalian reservoir host is also treated. This could be of great importance if control methods for canine leishmaniasis included extensive treatment of the domestic canine host. Treatment of canines has led to a reduction in parasite drug sensitivity as determined in assays on *L. infantum* isolates (73, 74). Current knowledge of the epidemiology and transmission of leishmaniasis suggests that the spread of acquired drug resistance is not a factor to be considered in cutaneous leishmaniasis except in anthroponotic foci of *L. tropica*. However, it is a factor that requires consideration in *L. infantum* leishmaniasis, where transmission is from human to human by needle (40) and a factor of major importance in anthroponotic disease foci such as *L. donovani* in Bihar State, India (146). This does not mean that there is no selection of resistant parasites in zoonotic infections in animals (74) or in humans during long courses of treatment, especially in immunocompromised patients (62). Rather these events must be considered in relation to chances of transmission of resistant parasites to the wider human population. These factors must also be separated from observations that indicate that in zoonotic leishmaniasis there are populations of parasites that are highly insensitive to a drug, as determined in drug sensitivity assays on isolates (164). These populations probably have a highly stable "resistance" phenotype (and genotype) and are transmitted from host to host.

### Strategies Available To Combat Drug Resistance

**Monitoring drug resistance.** Improved methods to monitor drug resistance that determine either the (i) phenotypic 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 are required. There are problems with both approaches. First, the determination of drug sensitivity of clinical isolates is open to the criticism that pathogen adaptation from host to culture media immediately selects for a subpopulation of pathogens best suited for growth in that medium. The drug sensitivity of parasites must therefore be tested as soon as possible after isolation from the

patient using defined agreed protocols. Although promastigote assays are easiest and quickest, this assay is not predictive for pentavalent antimonials, and possibly not for other antileishmanials, for example, paromomycin, pentamidine, and miltefosine. The amastigote-macrophage assay is currently the only model able to correlate clinical response to the sensitivity of the isolate, as demonstrated in relation to pentavalent antimonials (80, 96). Axenic amastigotes are sensitive to antimonials but adaptation of isolates is both too selective and too lengthy a process to be used in this type of assay (58, 131). Second, the ability to develop molecular probes or PCR-based diagnostics to monitor the development and spread of drug resistance is severely limited by a lack of knowledge of the molecular and biochemical mechanisms of action and resistance of most antileishmanial drugs, especially in clinical isolates.

**Monitoring therapy.** The introduction of an oral drug for leishmaniasis offers advantages of improved compliance, self-administration, and reduced costs. In the phase IV trial for miltefosine, a 7-day supply is issued to patients who have to return to the clinic each week for examination and resupply. For drugs like miltefosine which have a long half-life and a propensity for selection of resistant forms, the monitoring of daily dosing and the completion of a course of treatment is essential. The directly observed treatment strategy for tuberculosis chemotherapy has been successfully introduced in India by the Revised National TB Control Programme in 1997 ([www.who.int/gtb/publications/globerep/index.html](http://www.who.int/gtb/publications/globerep/index.html)). The potential for use of a parallel system for the control of leishmaniasis, for both miltefosine now and possibly sitamaquine in the future, should be considered.

**Cost and distribution of drugs.** The approximate cost of treatment of a patient with VL in India is given in Table 2. Progressive failure of antimonial drug treatment, which is the only available drug treatment in the public health program in India, has driven most of the VL patients in India towards the private sector. The drugs, including antimonials, amphotericin B, and now miltefosine, can be bought over the counter without restriction on quantity. The cash-starved population buys antileishmanial drugs in installments, and most do not complete treatment (141) as disease symptoms are alleviated quickly.

Considering the cost of drugs, antimonials have been the only drugs that are barely affordable. Miltefosine, which is being used extensively in the private sector, is >6 times more expensive and it is not mandatory to buy the full course. This is likely to result in widespread underdosing, sharing of doses among patients, and ultimately emergence of resistance to this important and only oral antileishmanial compound. It has been suggested that, considering the inability of the majority of the population to purchase and complete a full course of the drug and the chaotic system of drug marketing, miltefosine should be withdrawn from the private sector and made available free through public and/or private health care providers to prolong the effective life of this important drug (145).

**Diagnostic methods.** The improvement in noninvasive serological diagnostic methods with high sensitivity and specificity, for example, DAT, K39, and Katex (urine dipstick) are a major advance in the control of leishmaniasis (20, 77). In the context of chemotherapy what is required is a noninvasive diagnostic kit that can be used to monitor drug response and determine



TABLE 2. Cost of treatments for VL in India

Parameter	Drug regimen <sup>a</sup>						
	SAG, 20 mg/kg/day	AB, 1 mg/kg/ alternate days	ABLC, 2 mg/kg/day	L-amB, 5 mg/kg	L-amB, 7.5 mg/kg	Miltefosine, 100 mg/daily	ABLC, 2 mg/kg/day
Duration of therapy (days)	30	30	5	1	1	28	7.5
Cost of drug (30 kg) (US\$)	21	58.5	675	600	900	145 + 3.5	450
Total hospital cost (US\$)	350	368	74	24	24	62 + 10	85
Initial cost (US\$)	371	426.5	749	624	924	220.5	535
Treatment failure <sup>b</sup> (%)	60	3	10	10	10	6	5
Final cost per patient <sup>c</sup> (US\$)	634.4	439	792.9	667.9	967.9	246.84	556.95

<sup>a</sup> SAG, sodium antimony gluconate; AB, amphotericin B; L-amB, liposomal amphotericin B; ABLC, amphotericin B lipid complex. Miltefosine costs are based upon observed therapy with the cost of supervision plus, for women in the child-bearing age group, the cost of contraception (for example, depot hormone preparation effective for 3 months). Cost of counselling estimated at US\$10 and contraception for 1-month treatment and 3-month posttreatment at US\$3.5.

<sup>b</sup> All treatment failure patients retreated with conventional amphotericin B.

<sup>c</sup> (Initial cost per patient × 100 patients) + (cost of retreating failures)/100.

cure in patients. Antibody levels do not always indicate active infection, vary between individuals, and are of no use in HIV/VL coinfection cases. Antigen detection is far more important for monitoring drug response; the further improvement of methods such as the Katex kit (123) might be of particular interest in this case.

The variation in species sensitivity has greatest clinical significance in Central and South America, where the distribution of *L. mexicana*, *L. amazonensis*, *L. panamensis*, *L. braziliensis*, and other members of these groups overlap. The distinctive amastigote and macrophage interaction of mexicana group parasites makes some level of diagnosis by microscopy feasible for trained staff. Molecular tools that have been developed need to be implemented to distinguish braziliensis group species.

**Combination therapies.** Drug combinations have proven to be an essential feature of antimicrobial treatment through design or use to (i) increase activity through use of compounds with synergistic or additive activity, (ii) prevent the emergence of drug resistance, (iii) lower required doses, reducing chances of toxic side effects and cost, or (iv) increase the spectrum of activity, for example, the use of an antileishmanial with either an anti-inflammatory or immunodulator in cutaneous leishmaniasis. Previous studies on drug combinations for VL, for example, allopurinol plus sodium stibogluconate (31) and paromomycin plus sodium stibogluconate (32, 111, 150), have aimed to improve efficacy.

The use of combinations to combat resistance has been well rehearsed in antimalarials; for example, with resistance due to point mutations it has been estimated that symptomatic individuals harbor up to about 1,012 parasites. If a target enzyme has a mutation rate of  $10^{-7}$ , the chance of resistance to a single agent developing is high, but the likelihood of developing resistance to two compounds with different targets is very low (160). Studies to identify such combinations are new for leishmaniasis; limited studies are under way to examine interactions between miltefosine with other antileishmanials to identify suitable combinations (38). Bryceson (23) advocated the examination of combinations of strong antileishmanials with "weak" drugs (for example, azoles); this is an approach also used in malaria treatment, for example, the inclusion of clindamycin or azithromycin in combinations. A combination therapy also needs to be evaluated for safety and optimized for either concomitant or sequential administration of component drugs.

**Resistance reversal agents.** The strategy to reverse resistance has long been discussed in relation to chloroquine resistance in *Plasmodium falciparum* and produced interesting experimental results without any clinical impact (158). In laboratory studies on *Leishmania* a series of sesquiterpenes have been shown to reverse drug resistance due to P-glycoproteins in an *L. tropica* clone (119). Another study suggested a strategy of inhibition of thiol levels by coadministration of antimony with an inhibitor of glutathione biosynthesis (30). It is, however, unlikely that these approaches will have any clinical relevance.

**New targets, new drugs.** There are few better ways to avoid drug resistance than to have an adequate armory of drugs with different targets and no cross-resistance. Although miltefosine has been approved for use in the treatment of VL in India, paromomycin is moving through phase III trials in India and Africa, sitamaquine remains in phase II development for leishmaniasis (37), and all these drugs have clear limitations of toxicity, long courses of treatment, or parenteral administration. More clearly defined criteria of the needs and target profiles for new drugs and new treatments are required.

## CONCLUSIONS

Variation in the efficacy of drugs in the treatment of leishmaniasis is frequently due to differences in drug sensitivity of *Leishmania* species, the immune status of the patient, or the pharmacokinetic properties of the drug. Most leishmaniasis is zoonotic, where acquired drug resistance is not an important consideration. In areas with anthroponotic visceral leishmaniasis, especially India, acquired resistance to pentavalent antimonials has occurred and effective monitoring of drug resistance is needed. No molecular markers of resistance are available for currently used antileishmanial drugs. The only reliable method for monitoring resistance of isolates is the technically demanding in vitro amastigote-macrophage model. New treatments for visceral leishmaniasis have been introduced and others are undergoing 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.

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