Expert Opinion

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New delivery strategies for the old pentavalent antimonial drugs

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Importance of the field: Pentavalent antimonials are the first-line drugs for treatment of the major tropical disease leishmaniasis. However, their use is limited by the need for daily parenteral administration, their severe side effects and treatment failures. As leishmaniasis belongs to the group of neglected diseases, the improvement of old drugs through new delivery approaches has more support than the development of new chemical entities.
Areas covered in this review: The review covers, from 1977 to the present, the progress achieved towards pharmaceutically acceptable liposome-based formulations of antimonials, identification of specific ligands for improved targeting of infected macrophages and new approaches for oral and topical delivery of antimonial drugs.
What the reader will gain: Insights into the most promising delivery strategies to improve antimonial therapy and the chemical basis and future directions for achieving innovative orally and topically effective formulations.
Take home message: The development of drug delivery strategies for the old pentavalent antimonials is a still growing and promising field, with expected innovations in the near future from improved knowledge of antimony chemistry.

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Keywords: antimony, cyclodextrin, dogs, immunochemotherapy, leishmaniasis, liposomes, meglumine antimoniate, microparticle, pentavalent antimonials, stibogluconate

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1. Introduction

Pentavalent antimonials, including meglumine antimoniate (MA) and sodium stibogluconate (SSG), have been used for more than half a century in the therapy of leishmaniasis. This complex of diseases is caused by hemoflagellate protozoans and includes a potentially fatal visceral form caused by *Leishmania (L.) donovani*, *infantum* or *chagasi* and cutaneous forms caused by other species of *Leishmania* (**Table 1**). Leishmaniases are endemic in 88 countries, 22 in the New World and 66 in the Old World, and affect mainly poor and marginalized populations [1]. It is transmitted to humans through the bite of sandflies. Wild and domesticated animals, and humans themselves, can act as a reservoir of infection. *Leishmania* parasite is found as a motile promastigote in the sandfly, it transforms into an amastigote when engulfed by host macrophages, and resides in the acidic environment of secondary lysosomes [1].

Even though pentavalent antimonials are still the first-line drugs in South America, North Africa, Turkey, Bangladesh, Nepal and India (except North Bihar) for treatment of all forms of human leishmaniasis [2], they have several limitations [3,4]. Pentavalent antimonial drugs have to be given parenterally, daily, for at least 3 weeks (typically, 20 mg of Sb/(kg day) for 20 – 30 days). Antimony therapy is often accompanied by local pain during intramuscular injections and by systemic side effects, requiring very careful medical supervision. Typical side effects include nausea, vomiting, weakness and myalgia, abdominal colic, diarrhea, skin rashes,

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Article highlights.

- General information is provided about the target disease, its causative organisms and its symptoms, the clinical use of pentavalent antimonials and its limitations, and the current therapeutic alternatives.
- Key information is provided about the chemistry of pentavalent antimonials, and the actual knowledge of their mode of action and their pharmacokinetics by parenteral and oral routes is summarized.
- The technological problems and progress in the preparation of liposome formulations of antimonial drugs, the evaluation of these formulations for passive and active targeting of the infection sites of visceral and cutaneous leishmaniasis and strategies based on the association of these formulations with other active agents are reviewed.
- The main conclusions of the studies performed with conjugates of pentavalent antimonials with starch microparticles and mannan are presented.
- The recent use of cyclodextrin to improve the activity of meglumine antimoniate by the oral route and the probable mechanisms underlying the enhanced oral drug effectiveness are described.
- The rationale and possible strategies for effective cutaneous delivery of pentavalent antimonials are discussed.

This box summarizes key points contained in the article.

hepatotoxicity and pancreatitis, together with the most severe cardiotoxicity. Drug unresponsiveness represents another important problem in the control of this disease [1,4]. This may be caused by the drug resistance of the parasite [5], upregulation of ABC transporters in host cells [6], or host immunodepression, as reported in HIV-positive patients [7]. All these factors contribute to compliance difficulties and, eventually, treatment failures.

Visceral leishmaniasis (VL) is also a widespread and potentially fatal disease of dogs in South America, the Middle East and the Mediterranean region [8]. As dogs infected with *L. chagasi* or *L. infantum* represent the main natural reservoir of VL in these regions, there is major interest in an effective therapy for these animals, with two distinct aims: effective disease control and business opportunity. Canine leishmaniasis has been treated most frequently with the drugs MA, allopurinol, amphotericin B, aminosidine or a combination of MA and allopurinol [9]. However, therapy with these drugs usually achieves temporary clinical improvement and does not prevent relapse of disease or eliminate parasite carriage.

In light of these limitations, the World Health Organization strongly recommends and supports research into new drugs against leishmaniasis [10]. However, a lack of commercial return from drug development and of political support in the case of neglected diseases such as leishmaniasis has resulted in insufficient funding and commitment from both public sector agencies and the pharmaceutical industry [10]. In this context, strategies based on the improvement of existing drugs have been more successful than those based on the design of new chemical entities. Much effort has been devoted to the development of oral and topical drug formulations. Recent advances include the development of more effective and safer delivery strategies for existing antileishmanial drugs, the use of drugs originally designed and evaluated for non-related diseases, and new drug combinations and therapeutic protocols [4]. Importantly, two new drugs have recently reached the market to treat VL: a liposomal formulation of amphotericin B (AmBisome®, Gilead Sciences Inc., CA, USA), with reduced side effects and improved pharmacokinetic properties [11,12], and miltefosine (Impavido®, Zentaris AG, Frankfurt, Germany), originally developed as anticancer drugs, for oral treatment of VL [13]. Both drugs produced remarkable cure rates (> 90%) in clinical trials against human VL. Opposite to the long conventional antimony or amphotericin B regimens, it is worth mentioning the 1- or 2-day AmBisome regimens (10 mg/(kg day)), found to be highly effective in Indian VL patients [14] or routinely used in most European VL patients [15], respectively. However, these new drugs also present some limitations. The high cost of AmBisome makes its large-scale use in developing countries problematic. Not only is miltefosine a teratogen and shows a narrow therapeutic window [13], but it also requires 28 days of treatment, which, being taken orally on an outpatient basis, is critically prone to low compliance and the development of drug resistance. Furthermore, neither AmBisome nor miltefosine led to parasitological cure in dogs with VL [16-18].

Efforts have also been devoted to improving the therapy with pentavalent antimonials, with the aim of reducing their toxicity, enhancing their efficacy and increasing their bioavailability by non-invasive routes [19]. As detailed in this review, the most promising strategies involve the use of drug carrier systems, mainly liposomes, for the targeting of antimony to infection sites of VL and innovative pentavalent antimony complexes with improved bioavailability by oral and topical routes.

This article first presents the actual knowledge on the chemistry and pharmacology of pentavalent antimonials and then critically evaluates the delivery strategies under current investigation for these old drugs, from both the technological and pharmacological points of view.

2. Pentavalent antimonials: chemistry and pharmacology

2.1 Chemistry and mechanism of action

The two main pentavalent antimonials in current clinical use are complexes of Sb(V) with *N*-methyl-D-glucamine (MA or Glucantime®, Sanofi Aventis Farmacêutica Ltda) or sodium gluconate (SSG or Pentostam®, GlaxoSmithKline UK). These complexes are highly water-soluble, being manufactured typically at 85 g/l of Sb.

Although the exact structure of these complexes remained unknown for decades, mainly because of the amorphous state

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Table 1. Simplified relationship between *Leishmania* species and the main clinical forms in humans.

Clinical form	Geographical distribution	Subgenus	Species
Cutaneous leishmaniasis	Old world	*Leishmania*	*L. (Leishmania) major*
			L. (L.) tropica
			L. (L.) aethiopica
	New world		*L. (L.) mexicana*
			L. (L.) amazonensis
			L. (L.) venezuelensis
Tegumentary and mucocutaneous leishmaniais	New world	*Viannia*	*L. (Viannia) braziliensis*
			L. (V.) colombiensis
			L. (V.) guyanensis
			L. (V.) panamensis
			L. (V.) peruviana
			L. (V.) lainsoni
			L. (V.) naiffi
			L. (V.) shawi
			L. (V.) lindenbergi
Visceral leishmaniasis	Old world	*Leishmania*	*L. (Leishmania) donovani*
			L. (L.) infantum
	New world		*L. (L.) chagasi*

of these compounds, the use of mass spectrometric approaches and NMR techniques allowed significant progress [20-23]. Electrospray ionization mass spectrometry analyses have shown the existence of a mixture of oligomeric structures with the general formula (ligand-Sb)$_n$-ligand, which dissociate into 1:1 ligand-Sb complex on dilution [23]. **Figure 1** shows the structures proposed for the 1:1 Sb-ligand complex of MA and SSG.

Interestingly, MA is capable of forming ternary complexes in aqueous solution with hydroxyl-containing ligands, such as ribonucleosides [24] and cyclodextrin [25], according to the general formula (meglumine-Sb-ligand). A remarkable property of these ternary complexes is their slow dissociation rate on dilution [24], and, therefore, their ability to act as a sustained release system of the MA drug. In principle, this property makes feasible the use of specific ligands to improve the delivery of MA to infected macrophages. Another interesting pentavalent antimony complex is Sb-guanosine, which forms a hydrogel as a consequence of base stacking involving 1:1 and 1:2 Sb-guanosine complexes [26].

The metabolism and mechanism of action of pentavalent antimonials against leishmaniasis remain poorly understood [4,19]. It is not clear whether the final active form of pentavalent antimonials is Sb(V) or Sb(III). It has been reported that part of Sb(V) is reduced *in vivo* into the more toxic Sb(III) [27-29], suggesting that Sb(V) may be a prodrug. Recent studies have also indicated that thiols may act as a reducing agent in this conversion [30-32]. On the other hand, the formation of stable complexes between adenine ribonucleoside and Sb(V) has been reported [33], suggesting the involvement of ribose-containing biomolecules in the mechanism of action of pentavalent antimonials.

The toxicity of pentavalent antimonials is often attributed to the more toxic Sb(III) produced by the *in vivo* reduction of Sb(V). This model is supported by the similarities of the side effects observed during pentavalent and trivalent antimonial therapies [4]. However, some of the side effects of pentavalent antimonial drugs may also be due to the antimoniate ion (dissociated Sb(V)), as suggested by the relatively high toxicity of this ion to host cells [34,35].

2.2 Pharmacokinetics of pentavalent antimonials

Pharmacokinetic studies of Sb(V) compounds (e.g., SSG or MA) were performed in patients with VL following intramuscular administration [36]. For both drugs, the data were best described by a two-compartment, three-term pharmacokinetic model representing an initial absorption phase with a mean half-life of 0.85 h, a rapid elimination phase with a mean half-life of 2.0 h, and a slow elimination phase with a mean half-life of 76 h. About 80 – 95% of the drug appears in the urine within 6 h [37]. Thus, the high frequency of dosing in the case of pentavalent antimonial drugs is related to their fast renal excretion. Moreover, the long duration of the treatment (30 days) is related to the slow accumulation of Sb in the human body. The slow terminal elimination phase was believed to result from *in vivo* conversion of pentavalent Sb to trivalent Sb, which could contribute to the toxicity associated with long-term high-dose therapy.

Pharmacokinetic studies of MA were also performed in dogs after parenteral and oral administration [38,39]. Following parenteral administration, the curves of plasma concentrations versus time were best described by a triexponential open model with a mean (s.d.) half-life $t_{1/2}$ alpha of 9.4 (4.4) min, a $t_{1/2}$ beta of 45.3 (4.5) min and a $t_{1/2}$ gamma of 618.0 (93.5) min [38]. When given orally, an initial drug absorption phase with mean half-life of 0.23 h was observed, followed by an elimination phase with half-life of 2.6 h. The drug bioavailability was low, ~ 10% [39]. This explains why treatment with conventional antimonials is performed parenterally.

3. Liposomal formulations

Liposomes have been investigated most extensively among various colloidal carriers. They are microscopic vesicles consisting of one or more concentric spheres of lipid bilayers separated by aqueous compartments. These spherical structures can have diameters ranging from 50 nm to 5 μm. The ability of liposomes to entrap both hydrophilic and hydrophobic drugs, their versatility and their amenability for surface modification are the chief factors responsible for their popularity in drug delivery research.

3.1 Technological aspects of liposome production

The complexity of liposomal drug formulations, when compared with conventional drugs, implies that not only

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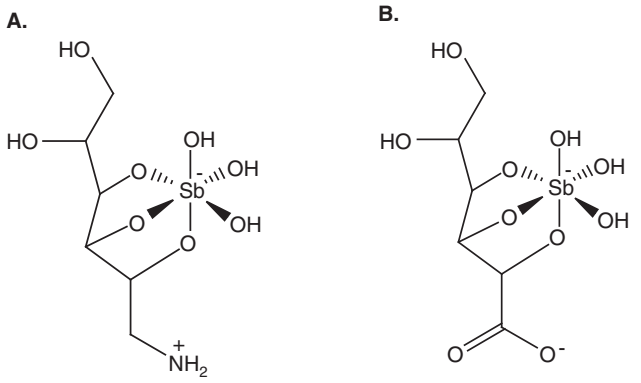

Figure 1. Proposed structural formula for (A) meglumine antimoniate and (B) stibogluconate in aqueous solution.
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pharmacological issues but also technological problems related to their production must be considered in the rational design of such formulations. In that sense, a critical point is the choice of the method of preparation of the liposome-based formulation. The level of difficulty also increases considerably when the drug to be encapsulated is water-soluble, as the achievement of high drug encapsulation efficiency and long-term stability during storage turns out to be a great challenge.

Table 2 summarizes the main methods proposed for the encapsulation of antimonial drugs in liposomes. Until recently, essentially two different methods had been used. One method consisted of the hydration of a thin film of lipids with a solution of the drug, followed or not by sonication of the liposome suspension [40]. The other method, known as the reverse-phase evaporation procedure, involved the formation of a water-in-oil emulsion using the drug solution as aqueous phase, followed by evaporation of the organic solvent, resulting in a phase change and the formation of a vesicle suspension [41]. The main advantage of the latter method, compared with the former, is that it yields higher efficiencies of drug encapsulation and higher ratios of encapsulated drug to lipid. These characteristics mean that a lower quantity of lipid has to be injected in order to introduce the same quantity of antimonial, which makes the treatment safer and more economical. Nevertheless, liposomes prepared by the reverse-phase evaporation procedure may be toxic at high doses owing to unavoidable residual traces of organic solvent in the final liposome formulation. Furthermore, the reverse-phase evaporation method is not appropriate for large-scale industrial production. A significant limitation of both methods is that the resulting liposome preparations could be stored only as aqueous suspensions. In this condition, however, a significant leakage of the drug occurred with time from the internal to the external continuous aqueous phase [40,41]. For example, a typical liposomal formulation prepared by the reverse-phase evaporation procedure released > 26 – 48% of the originally encapsulated drug when stored for 7 weeks at 25°C [41].

Typical lipid compositions of the liposome formulations are mixtures of natural or synthetic phosphatidylcholine (PC) and cholesterol (CHOL). The inclusion of negatively charged phospholipid, such as dicetylphosphate (DCP), was found to improve the encapsulation efficiency of MA and consistently to promote efficacious formulation [42]. Phosphatidylcholines with long and saturated hydrocarbon chains, such as dipalmitoylphosphatidylcholine (DPPC), were often preferred because of their lower susceptibility to oxidation and to the lower permeability of their membrane. As illustrated in **Table 2**, the size and lamellarity of the vesicles have a strong influence on the encapsulation efficiency of water-soluble drugs. The general tendency is that vesicles with larger size or reduced lamellarity have higher internal aqueous volume and drug loading efficiency.

Shelf-life of the vesicle suspension, chemical stability of liposome constituents and cost of lipids are important issues for the industrial production of phospholipid-based formulations. In this context, chemically stable non-ionic surfactants, such as monoalkyl or dialkyl polyoxyethylene ether or sorbitan esters, have been proposed as a low-cost alternative to phospholipids for the preparation of vesicles called niosomes [43,44]. Another potential advantage of niosomes is that their method of preparation may be more suitable for large-scale production [45].

New processes for the preparation of liposomal MA were introduced recently, with significant technological advantages over previously described methods [46-49]. Those are based on the preparation of negatively charged empty liposomes in the freeze-dried state and the subsequent rehydration of the lyophilized liposomes with an aqueous solution of the antimonial drug (**Figure 2**). Of significant advantage, liposomes may be stored as pre-formed freeze-dried empty vesicles and rehydration may be performed just before use. This circumvents the stability problems arising from the long-term storage of liposomes in the presence of the water-soluble drug. As illustrated in **Figure 2**, two different liposomal formulations, with mean hydrodynamic vesicle diameters of 1200 and 400 nm, were obtained [49].

In the case of liposome-encapsulated antimonial drugs, owing to the marked increase of treatment efficacy and the consequent administration of much lower dose of Sb, the separation step of liposomal drug from non-encapsulated drug can be omitted in the process of liposome formulation, thereby simplifying the preparation process.

3.2 Conventional liposomes for passive targeting of infections sites of VL

During 1977 – 78, groups in London, Liverpool and Washington showed that pentavalent antimonials, when encapsulated in conventional liposomes, that is, made from PC and CHOL, were 200 – 700-fold more effective than the free drug when comparing a single intravenous (i.v.) dose against *L. donovani* in a rodent model of VL [50-52].

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Table 2. Different formulations of antimonial drugs in liposomes.

Type of liposomes (preparation method)	Lipid composition (mean vesicle diameter)	Loading efficiency	Sb/lipid ratio (w/w)	Condition of storage	Ref.
Multilamellar vesicles (thin film hydration)	DPPC/CHOL/DCP PC/CHOL/PA (> 1000 nm)	8%	< 0.5	Aqueous suspension	[40,50]
Small unilamellar vesicles (ultrasonication)	PC/CHOL/DCP (< 150 nm)	< 10%	< 0.1	Aqueous suspension	[52]
Oligolamellar vesicles (reverse-phase evaporation)	DPPC/CHOL/DCP (500 nm)	30 – 50%	3 – 6	Aqueous suspension	[41,53]
Niosomes (lipid melting + hydration + homogeneization)	Non-ionic surfactant/ CHOL/DCP (100 – 600 nm)	10 – 45%	< 2	Aqueous suspension or freeze-dried form	[44,60]
Large-sized oligolamellar vesicles (hydration of freeze-dried empty liposomes)	DSPC/CHOL/DCP (> 1000 nm)	30 – 50%	3 – 6	Freeze-dried form	[46]
Medium-sized oligolamellar vesicles (hydration of freeze-dried empty liposomes with sucrose)	DSPC/CHOL/DCP (250 – 450 nm)	30 – 50%	3 – 6	Freeze-dried form	[47,49]

CHOL: Cholesterol; DCP: Dicetylphosphate; DPPC: Dipalmitoylphosphatidylcholine; DSPC: Distearoylphosphatidylcholine; PA: Phosphatidic acid; PC: Egg-lecithin.

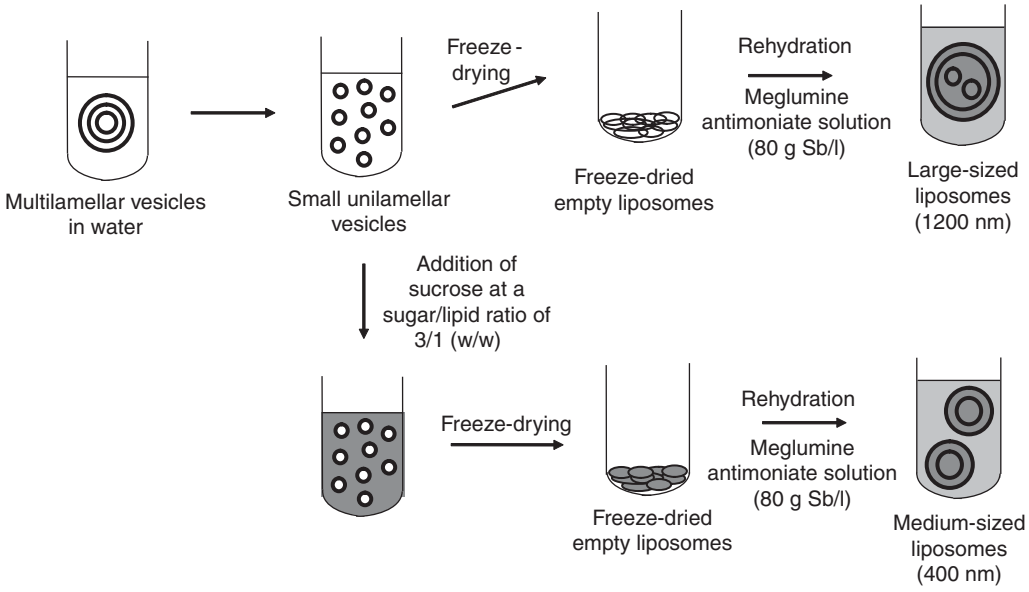

Figure 2. Processes used for the preparation of meglumine antimoniate-containing liposomes of different size.
Reproduced with permission from [19].
CHOL: Cholesterol; DCP: Dicetylphosphate; DSPC: Distearoylphosphatidylcholine.

As illustrated in **Figure 3**, this spectacular effect of liposome encapsulation was attributed to the sustained drug release properties of liposomes and to their natural tendency to be cleared from the circulation by the fixed macrophages of the mononuclear phagocyte system (MPS), mainly the liver, spleen and bone marrow, which are the major sites of parasite infection of VL. Indeed, following liposomal delivery, antimony levels in the liver and spleen of mice were 5 – 20-fold higher than that achieved by free drug and were maintained for up to 14 days [50,53]. Moreover, ultrastructural studies revealed the lysosomotropic route of the loaded liposomes to the required intracellular site – the parasitophorous vacuole [54,55].

Later on, the marked impact of liposome encapsulation on the efficacy of pentavalent antimonials was confirmed with *L. chagasi* and *L. infantum* experimental infections [46,56] and

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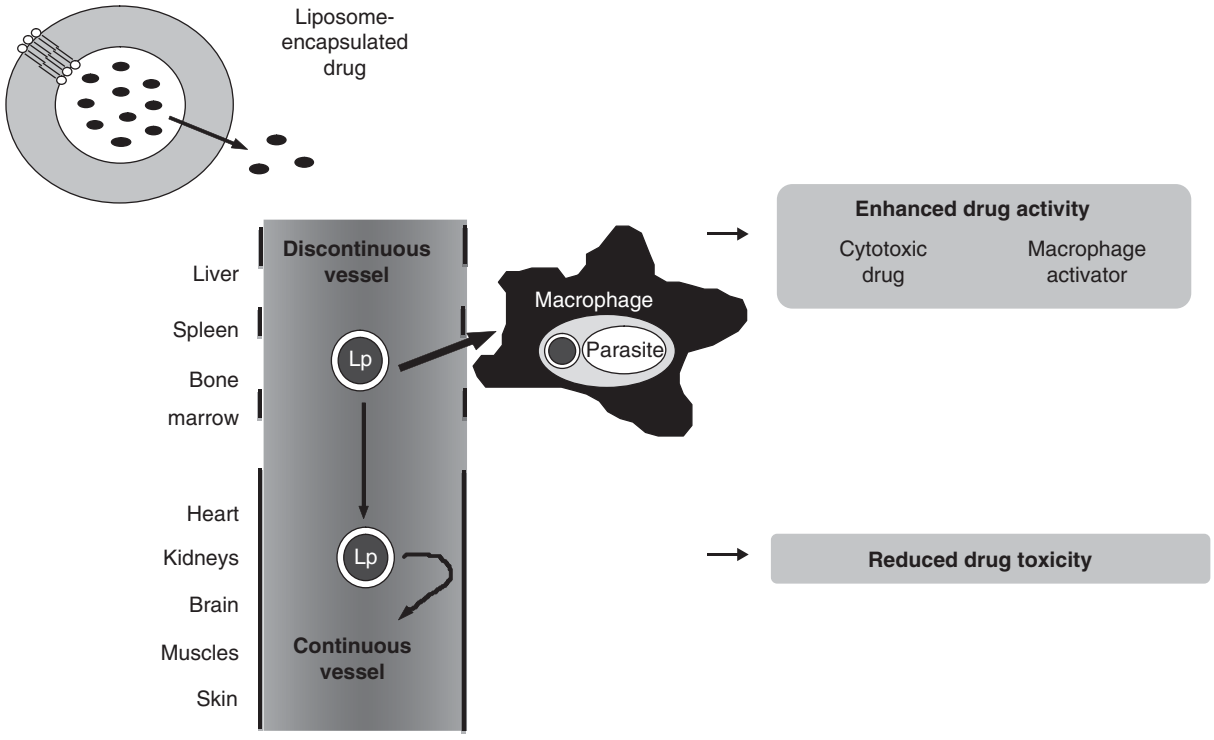

Figure 3. Illustration of the fate of liposome-encapsulated drug after intravenous administration.
Lp: Liposomes.

using niosomes instead of liposomes [44,57]. On the other hand, the effectiveness of liposomal MA was found to depend on the virulence of infection, the activity being greater in less virulent infections [58].

As illustrated in **Figure 3**, liposome encapsulation also reduces the drug concentration in organs with continuous capillaries, such as the heart and kidneys, allowing a decrease of drug cardiotoxicity and nephrotoxicity. On the other hand, the high drug targeting of the liver is expected to result in enhanced toxicity compared with the free drug at the same dose of Sb. Taking into account the toxicity of Sb owing to its concentration in liver cells, an overall 35 – 40-fold improvement in the therapeutic index was estimated [59]. Following a single intravenous dose of SSG-containing niosomes in healthy beagle dogs at 10 mg Sb/kg, serum hepatic enzyme levels increased and iron levels decreased considerably over the physiological range of values [60], evidencing liver dysfunction. On the other hand, no change in serum markers of hepatic function was observed in mongrel dogs with VL following a multiple dose regimen with liposome-encapsulated MA given as 4 doses of 6.5 mg Sb/kg (body weight) with 4-day intervals [61]. In both studies, however, transitory adverse reactions, including prostration, defecation, tachypnea and sialorrhea, were observed during the first hour after each injection of the vesicular formulations to dogs. As the same effects were also observed with empty liposomes, they were independent of the metal. Indeed, such lipid vesicle-induced acute effects have been described previously as complement-mediated pseudoallergic reactions [62].

3.2.1 *Influence of vesicle characteristics*

Table 3 summarizes the experimental evidence for the influence of vesicle surface charge and vesicle size on the pharmacokinetics and antileishmanial efficacy of liposome-encapsulated antimonial drugs.

Based on parasite suppression in the liver of hamsters experimentally infected with *L. donovani*, positively charged egg PC liposomes containing MA were found to be less effective than negatively charged ones [51]. By contrast, positively and negatively charged sphingomyelin liposomes were equally effective. Surprisingly, liposomes containing the negatively charged phosphatidylserine were among the less effective preparations. In another study, both negatively charged and neutral vesicles were found to be equally effective [57]. More recently, however, Pal *et al.* argued that positively charged egg PC liposomes containing SSG reduced parasite burden in both the liver and the spleen of infected mice [63], in contrast to negatively charged liposomes, which were effective only against liver parasites [64]. Nevertheless, the possible influence of the vesicle size was not considered in this study. Indeed, small liposomes (mean diameter < 100 nm), containing an antimonial drug, were more effective than vesicles of larger size in reducing the number of *Leishmania* parasites in the bone marrow and

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Table 3. Parameters influencing the properties of liposome-encapsulated antimonial drugs.

Parameter	Evaluated property	Results	Ref.
Vesicle size	Loading efficiency	Larger vesicles have greater internal aqueous volume and drug loading efficiency	
	Pharmacokinetics	Medium-sized liposomes targeted bone marrow more effectively than large-sized ones in dogs with VL	[49]
	Antileishmanial activity	Medium-sized liposomes promoted significant suppression of parasites in dogs with VL, contrary to large-sized liposomes Only very small liposomes (diameter < 100 nm) were effective in reducing the number of *Leishmania* parasites in the spleen and bone marrow of mice with VL	[61,65,66]
Surface charge	Loading efficiency	Negatively charged vesicles were claimed to encapsulate antimonial drugs more effectively than neutral or positively charged vesicles	[50,51]
	Antileishmanial activity	Negatively charged PC liposomes are more effective than positively charged ones against liver parasites in mice with VL Positively charged PC liposomes promote suppression of splenic parasites in mice, contrary to negatively charged liposomes	[51,63]
Ligand for macrophage surface receptor	Antileishmanial activity	Mannose-grafted liposomes showed enhanced drug effectiveness in mice with VL, compared with conventional liposomes Tuftsin-grafted liposomes showed enhanced drug effectiveness in mice with VL, compared with conventional liposomes	[71,78]
Macrophage activator	Antileishmanial activity	Co-incorporation of gamma-interferon or tuftsin enhanced the drug effectiveness in mice with VL, compared with liposome containing only antimony	[78,86]

PC: Phosphatidylcholine; VL: Visceral leishmaniasis.

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spleen of mice [65]. Furthermore, treatment of naturally infected mongrel dogs with large liposomes (mean diameter > 1000 nm) with 4 doses of ~ 6 mg Sb/kg did not produce significant reduction of parasite burden in the liver, spleen and bone marrow when compared with untreated animals [66]. This is in contrast to the treatment of infected dogs with medium-sized liposomes (mean diameter = 400 nm) at about the same dosage that produced significant parasite suppression in the liver and spleen [61]. Interestingly, the level of antimony achieved in the bone marrow of infected dogs was two- to threefold higher from medium-sized liposomes than from large ones [49].

With respect to the influence of membrane fluidity, the most consistently effective liposomes were those containing saturated long-chain PC and CHOL [51], that is, those with the more rigid and less permeable membrane. Niosomal and liposomal antimony-containing formulations were equiactive and both increased drug efficacy by an order of magnitude compared with that of free drug [57].

Until now, there has been no clear picture as to how the surface charge and size of liposomes affect their interaction with the macrophages and their distribution *in vivo*. The capture of liposomes by macrophages is favored by the process of opsonization, which occurs just after their contact with blood components. There is experimental evidence that liposomes of differing morphologies (size and lamellarity) and surface characteristics may attract different arrays of plasma proteins, called 'opsonins', the content and conformation of which may account for the different pattern in the rate and site of vesicle clearance from the blood [67].

Another important step after liposome capture is the processing of liposomes by macrophages and, more specifically, the degradation of phospholipid by lysosomal lipases and the release of encapsulated drug within the phagolysosomes. Membrane fluidity was found to influence the rate of drug release, the more rigid membranes being the more resistant to destabilization by serum components and the less susceptible to lysosomal degradation [67].

3.3 Liposomes for active targeting

Macrophages, in which *Leishmania* parasites reside, possess various receptors such as Fc receptors, complement, fibronectin, lipoprotein, mannosyl, galactosyl and scavenger receptors (SRs) [68]. These macrophage surface receptors determine the control of activities such as activation, recognition, endocytosis and secretion [69].

Many approaches for targeting the drugs to the macrophages have been developed, which are largely represented by colloidal carriers. Although these carriers show natural affinity towards the macrophages and are passively targeted to them, inclusion of the macrophage receptor-specific ligands may significantly enhance the rate and extent of their uptake by the macrophages. Thus, macrophage-specific ligands, such as mannose, tuftsin residues and phosphatidylserine, have been incorporated into liposomes to enhance their active uptake by macrophages.

3.3.1 *Sugar-bearing liposomes*

Sugar-bearing liposomes were designed to achieve further improvement in macrophage targeting of antileishmanial

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agents as the macrophage surface contains receptors that recognize terminal galactose, mannose, fucose or glucose residues of glycosides [70]. In this context, efficacy of urea-stibamine in free, liposomal and mannose-grafted liposomal forms was tested against experimental VL in a hamster model. Mannose-grafted liposomes showed higher antileishmanial activity and were more effective at transporting the drug to the macrophages as compared with glucose-grafted liposomes, conventional liposomes and free drug [71,72]. A possible limitation to this approach, however, is the downregulation of sugar receptors in macrophages following infection with *Leishmania* [73].

3.3.2 Phosphatidylserine-containing liposomes

Macrophages express a range of cell surface glycoproteins, namely SRs, which are able to bind modified lipoproteins, senescent and apoptotic cells, proteins, polysaccharides and a range of polyanionic molecules. The broad ligand specificity of class B scavenger receptors (CD36 and SR-BI) and class A (MARCO) makes them attractive candidates for mediating the binding and uptake of liposomes containing negatively charged phospholipids, such as phosphatidylserine [74].

Phosphatidylserine-containing liposomes were investigated as an attempt to take advantage of the upregulation of macrophage SR mRNA during the initial steps of *L. chagasi* infection [74]. Surprisingly, however, the uptake of Sb from these liposomes was reduced in infected macrophages compared with non-infected ones, suggesting a low metabolic rate in infected macrophages.

3.3.3 Tuftsin-grafted liposomes

Tuftsins have been used for improving the targeting of antileishmanial drugs owing to their property of preferential binding to cells of the MPS system [75]. Tuftsin is a basic tetrapeptide (Thr-Lys-Pro-Arg) that was found to reveal several biological functions associated with the immune system. It is generated in the body from a specific cytophilic fraction of the protein (leukokinin) through a two-step enzymatic processing mechanism [76]. Importantly, the tetrapeptide enhances the phagocytic activity of monocytes and macrophages and acts as an immunomodulator by activating MPS nonspecifically against infections [77].

Guru *et al.* evaluated the potential of tuftsin-bearing liposomes (composed of egg phosphatidylcholine and cholesterol) for delivery of SSG in *L. donovani*-infected hamsters [78]. Interestingly, tuftsin-bearing SSG liposomes showed significantly greater suppression of splenic parasites than that obtained with free SSG or tuftsin-free liposomal SSG.

3.4 Association of liposomal formulations with other active agents

3.4.1 Stearylamine-containing liposomes

Dey *et al.* reported the activity of liposomes comprising egg PC and stearylamine (SA) against *L. donovani* parasites [79]. Both promastigotes and intracellular amastigotes *in vitro* and *in vivo* were susceptible to SA-egg PC liposomes. A single dose of 55 mg of SA-egg PC liposomes/animal could significantly reduce the hepatic parasite burden by 85 and 68% against recent and established experimental VL. On the other hand, the level of serum glutamine pyruvate transaminase was increased at day 15, indicating a transitory alteration in hepatic function.

In a subsequent study, SA-egg PC liposomes containing SSG were evaluated for elimination of *L. donovani* parasites from the liver and spleen of BALB/c mice with established and chronic infections [63]. A synergistic activity of SSG entrapped in SA-egg PC liposomes was claimed in both *in vitro* and *in vivo* models of VL, even though comparison with pure PC liposomes (SA-free) was not presented.

3.4.2 Immunochemotherapy

The antileishmanial effectiveness of antimonial drugs depends on the host immune status and, more specifically, on the level of activation of infected macrophages [4]. The intracellular localization of *Leishmania* parasite makes the association of pentavalent antimonials with macrophage activators an extremely promising therapeutic strategy for leishmaniasis [80]. Macrophage activation during treatment with antimonial drugs allowed effective treatment with reduction of applied antimony dose. This was demonstrated using the nonspecific activator gamma-interferon [81], granulocyte-macrophage colony-stimulating factor (GM-CSF) [82], liposome-encapsulated muramyl dipeptide [83] and specific Th1 response-inducing *Leishmania* antigen vaccine [84,85].

So far, just one study has reported the efficacy of liposomal antimonial drug in the presence of macrophage activator. Gamma-interferon was combined with the antimonial drug in the same multilamellar vesicles. Treatment of *L. donovani*-infected mice with this formulation resulted in nearly complete parasite elimination, whereas treatment with the free drug slightly reduced the parasite burden only in the liver [86].

3.5 Advanced studies of liposomal Sb in dogs with VL

As dogs infected with *L. chagasi* or *L. infantum* are the main natural reservoir of VL in Latin America and in the Mediterranean region and respond poorly to conventional antileishmanial therapies [8], much effort has been devoted to the achievement of an effective liposome formulation in these animals.

Table 4 summarizes the studies performed so far with liposome-encapsulated antimonials in dogs with VL. Chapman *et al.* determined the antileishmanial efficacy of MA-containing large multilamellar vesicles in mongrel dogs experimentally infected with *L. donovani* amastigotes [87]. The antileishmanial agents (encapsulated and free MA) were given once daily, intravenously, for 1, 4, or 10 consecutive days beginning the twelfth day after inoculation. The dogs were killed 3 or 4 days after completion of therapy, and

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Table 4. Assays of liposome-encapsulated antimonial drugs in dogs with VL.

Type of liposomes (preparation method)	Lipid composition (mean diameter)	Dosage	Infection	Main results	Ref.
Multilamellar vesicles (thin lipid film hydration)	DPPC/CHOL/DCP (> 1000 nm)	1 i.v. dose of 0.6 mg Sb/kg or 4 i.v. doses of 1.94 mg Sb/(kg day)	Mongrel dogs experimentally infected with *L. donovani*	89 and 95.8% suppression of splenic parasites after 1 dose (0.6 mg Sb/kg) and 4 doses (1.94 mg Sb/(kg day))	[87]
Small unilamellar vesicles (homogenization)	PC/sodium cholate (10 nm)	10 doses of 9.8 mg Sb/(kg dose) (2 i.v. first doses, 8 s.c. doses)	Beagle dogs experimentally infected with *L. infantum*	Long-term efficacy for 1 year on the basis of gamma-globulin levels	[88]
Large-sized oligolamellar vesicles (hydration of freeze-dried empty liposomes)	DSPC/CHOL/DCP (> 1000 nm)	4 i.v. doses of 6 mg Sb/kg (4-day interval)	Mongrel dogs naturally infected with *L. chagasi*	Reduction of the number of positive dogs based on evaluation of bone marrow parasite burden 30 days after treatment, compared with untreated group; no significant reduction in parasite load in spleen, liver and bone marrow 5 months after treatment	[66]
Medium-sized oligolamellar vesicles (hydration of freeze-dried empty liposomes with sucrose)	DSPC/CHOL/DCP (250 – 450 nm)	4 i.v. doses of 6 mg Sb/kg (4-day interval)	Mongrel dogs naturally infected with *L. chagasi*	Significant (95.7%) parasite suppression (liver, spleen, lymph nodes) and reduction of infectivity to sandflies, 5 months after treatment, when compared with untreated group	[61]

CHOL: Cholesterol; DCP: Dicetylphosphate; DPPC: Dipalmitoylphosphatidylcholine; DSPC: Distearoylphosphatidylcholine; i.v.: Intravenous; PC: Egg-lecithin; s.c.: Subcutaneous.

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parasites in the spleens were quantified. A single injection of liposomal MA (0.61 mg Sb/kg (body weight)) resulted in 89% suppression and 4 consecutive daily injections of the liposome formulation (1.94 mg Sb/(kg day)) resulted in 95.8% suppression of splenic parasites. The liposome-encapsulated drug was estimated to be > 700 times more efficacious than the unencapsulated drug, based on a single dose treatment.

Valladares *et al.* reported the efficacy of MA-containing small-sized liposomes in beagle dogs experimentally infected with *L. infantum* [88]. Dogs with VL were treated daily for 10 days with a liposome formulation of MA at 9.8 mg Sb/(kg day) (the first 2 doses administered intravenously and the subsequent 8 doses given subcutaneously). This treatment was repeated after a resting period of 10 days. Long-term efficacy for at least 1 year was claimed on the basis of gamma-globulin levels.

A liposome formulation of MA consisting of large vesicles (1200 nm mean diameter) was evaluated in mongrel dogs naturally infected with *L. chagasi* [66]. Following a multiple-dose regimen (4 doses of 6 mg Sb/kg (body weight) with 4-day intervals), this formulation resulted in a significantly lower number of positive dogs based on an evaluation of bone marrow parasite burden, 30 days after treatment, compared with the group treated with empty liposomes and an untreated group. However, evaluation of the parasite burden in the liver, spleen and bone marrow 5 months after treatment revealed no significant parasite suppression in the group treated with liposomal MA.

Another liposome formulation of MA was obtained consisting of medium-sized vesicles (400 nm mean diameter), and its pharmacokinetics was evaluated in mongrel dogs with natural VL. This formulation was found to target more effectively the bone marrow of infected dogs than a similar formulation of MA but in large liposomes [49]. The antileishmanial activity of this formulation was then investigated in naturally infected dogs after treatment with 4 doses of 6.5 mg Sb/kg (4-day intervals) [61]. Parasite suppression > 95% was demonstrated in cervical lymph nodes, liver and spleen of dogs 5 months after treatment. Feeding of *Lutzomyia longipalpis* phlebotomines on dogs treated with the liposomal drug, 5 months after treatment, resulted in a significant reduction of sandfly infection, compared with feeding on control animals. This study was the first report of both long-term parasite suppression and reduction of infectivity to sandflies in naturally infected dogs following treatment with a liposome-encapsulated drug. Importantly, this was achieved using a 20-fold lower cumulative dose of Sb compared with that used for conventional antimonial treatment. Nevertheless, this treatment did not lead to a parasitological cure of infected dogs, as *Leishmania* parasites could still be detected in their bone marrow. In the same study, safety evaluations in dogs treated with the liposome formulations indicated no change in serum markers of hepatic function (aspartate

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aminotransferase, alkaline phosphatase, alanine aminotransferase, total bilirubins) and renal function (urea, creatinine). The hemogram parameters also did not show any significant alteration on treatment [61].

3.6 Potential for treatment of cutaneous leishmaniasis

Treatment of cutaneous leishmaniasis (CL) by antimonial drugs when administered by various routes to mice infected with *Leishmania major* or *Leishmania amazonensis* was found to be enhanced, relative to the free drug, by entrapment of these compounds within liposomes [89]. SSG-loaded liposomes given intravenously were more effective at reducing the growth of a cutaneous lesion when administered after the nodule had begun to develop, rather than at the time of inoculation of the parasites. In contrast to the intravenous route, liposomes administered subcutaneously at the inoculation site were effective only if given at the time of infection. It has been estimated that liposome-encapsulated antimonial was 10 – 15-fold more effective than free drug [90]. The surprising efficacy against CL of the intravenous treatment with liposomal drug was attributed to migration of blood monocytes, containing recently endocytosed liposome-entrapped drug, to the lesion. In all cases, however, the activity of liposomal drug was considered minimal, causing a suppression of lesion growth rather than a cure [90].

4. Polymer-based drug delivery systems

Polyacryl starch microparticles containing covalently bound SSG have been evaluated in mice experimentally infected with *L. donovani* [91]. The carrier form was up to 100-fold more effective than the free form. Empty microparticles had no effect on liver parasite burdens and the enhanced *in vivo* antileishmanial activity of the carrier form of the drug was attributed to passive drug delivery to the infected liver as a result of the colloidal character of the carrier.

As another approach to targeting the mannosyl macrophage surface receptor, a pentavalent antimony-mannan conjugate was prepared. When evaluated in a murine model of VL, the drug conjugate was 10-fold more potent than SSG [92]. Liver antimony concentrations were sixfold higher after Sb(V)-mannan therapy compared with a dose of SSG that was equipotent in reducing liver parasite burdens. Murine toxicity of Sb(V)-mannan was variable, with a 50% lethal dose for one preparation that was modestly higher than the concentration that killed 90% of the parasites.

5. Cyclodextrin-based oral formulation

The need for parenteral administration and the side effects of conventional pentavalent antimonials lead to non-compliance of the dose regimen and consequently treatment failure. It is therefore desirable to develop methods for enhancing the bioavailability of these drugs by the oral route.

The association of drugs to carrier systems is a feasible strategy to improve their absorption by the oral route. Cyclodextrins, which are cyclic oligosaccharides composed of glucose units joined through α-1,4 glucosidic bonds, are well known in recognition chemistry as molecular hosts capable of including, with a degree of selectivity, water-insoluble guest molecules by means of non-covalent interactions within their hydrophobic cavity. Thus, this carrier has been widely used to improve the oral bioavailability of water-insoluble drugs, owing to the enhancement of the drug apparent solubility and dissolution rate [93,94].

Demicheli *et al.* reported recently that the composition comprising MA and β-cyclodextrin (β-CD), prepared in equimolar amounts of Sb and β-CD, enhanced the absorption of Sb by the oral route and rendered the antimonial drug orally active in a murine model of CL [95]. Antimony concentrations in the plasma of mice were about three times higher for the association compound than for free MA, when given orally. Antileishmanial efficacy was evaluated in BALB/c mice infected with *L. amazonensis*. Animals treated daily with MA/β-CD composition (32 mg Sb/kg for 12 days) by the oral route developed significantly smaller lesions when compared with those treated with threefold higher doses of MA (120 mg Sb/kg for 12 days). The effectiveness of the composition given orally was equivalent to that of MA given intraperitoneally at a twofold higher antimony dose. The antileishmanial efficacy of the complex was confirmed by the significantly lower parasite load in the lesions of treated animals when compared with saline controls [95].

On the other hand, when MA/β-CD was evaluated in BALB/c mice infected with *L. donovani* (45 mg Sb/(kg day) by gavage for 10 days), no significant suppression of liver parasites was observed (V Yardley, unpublished results). The discrepancies in MA/β-CD effectiveness between the CL and VL models are probably owing to the higher sensitivity of the former model to antimonial drugs.

As MA is highly soluble in water and does not interact with the hydrophobic cavity of β-CD, a non-classical mechanism is responsible for the enhanced oral effectiveness of this drug. Investigation of the mechanism involved must take into account the process used to prepare the formulation. The first step consists of the heating of the antimonial+β-CD mixture in water and the second step involves the freeze-drying of the resulting solution.

The ability of β-CD to improve serum Sb levels was attributed, in part, to the fact that the heating of the antimonial+β-CD mixture induced the depolymerization of the antimonial drug from high-molecular-mass Sb complexes into 1:1 Sb-meglumine complex [25]. Characterization of the heated antimonial+β-CD mixture, using circular dichroism and electrospray ionization mass spectrometry, also indicated the formation of a ternary meglumine-Sb-β-CD complex showing stability constant of 100 l/mol [25].

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As the heated antimonial+β-CD mixture promoted significantly lower serum Sb levels when compared with the final composition [96], the freeze-drying process contributed also to the high absorption of Sb by the oral route. Physicochemical characterization of the final lyophilized antimonial/β-CD composition indicated the formation of supramolecular nanoassemblies with a mean hydrodynamic diameter of ~ 200 nm, comprising 1:2:1, 2:2:1 and 2:2:2 meglumine-Sb-β-CD complexes [96]. Another important observation was the ability of the MA/β-CD composition to act as a sustained release system of the MA drug, suggesting that this property may result in prolongation of drug absorption in the gastrointestinal tract.

Despite these very promising results, the low water solubility of the 1:1 MA/β-CD composition appeared to be a limitation for its application at higher dose and in large mammals, such as dogs and humans [39]. Indeed, to achieve therapeutic concentrations of Sb, large volumes and large quantities of β-CD have to be lyophilized and administered. Such conditions increase the cost of treatment and the incidence of side effects related to β-CD. In this context, some strategies were investigated to improve the solubility of MA/β-CD composition. It was observed that increasing the MA/β-CD ratio in the composition markedly improved its solubility in water [39]. Pharmacokinetic study of the 7:1 MA/β-CD composition following intragastric administration to beagle dogs at 100 mg Sb/kg indicated that β-CD enhances the oral bioavailability of MA from 10 to 15% and its plasma mean residence time from 4.1 to 6.8 h [39]. Evidence was obtained by ^{1}H NMR that Sb is absorbed in the form of MA and remains essentially under the pentavalent oxidation state. This study established the sustained drug release property of 7:1 MA/β-CD composition and its ability to enhance the plasma mean residence time of MA after oral administration.

Figure 4 illustrates the model proposed for the enhanced delivery of Sb from the antimonial/β-CD composition by the oral route. Accordingly, the slow release property of antimonial/β-CD nanoassemblies would allow for their migration along the gastrointestinal tract. Antimonial/β-CD nanoassemblies would then release slowly the antimonial drug in the form of 1:1 Sb-meglumine complex, which would readily permeate by simple diffusion across the intestinal epithelium.

6. Topical formulations for cutaneous leishmaniasis

The efficacy of intralesional MA in the treatment of CL [97] suggests that antimonials are promising drugs for topical treatment of CL. However, topical formulations of SSG did not show satisfactory activity against CL [98]. This result may be attributed to the high water solubility of this pentavalent antimonial and to its low permeability coefficient across biological membranes. In this context, the use of Sb(V) complexes with cyclodextrin [25] or other amphiphilic ligands represents a promising approach to improve the efficacy of pentavalent antimonials in topical formulation. Of special interest is the Sb(V)-guanosine hydrogel [26], which was found to be highly effective against intracellular *Leishmania* amastigote [35].

7. Conclusion

Clinical use of pentavalent antimonials against leishmaniasis is severely limited by the need for repeated parenteral administrations, their toxic side effects and treatment failures. As detailed in this review, liposomes were successfully used for passive and active targeting of Sb to the infection sites of VL. Solutions based on the freeze-drying technique or the use of non-ionic surfactants were proposed in response to the technological difficulties of long-term storage of liposome formulations and of scaling up their process of preparation. Liposome-mediated delivery of antimonial drug to VL infection sites was found to be highly effective with conventional liposomes and was improved further through specific targeting of macrophage surface receptors using mannose or tuftsin ligand. On the other hand, ligand-targeted liposomes seem to be captured less avidly by infected macrophages. The strategy based on the combination of liposome-encapsulated antimonial drugs with macrophage-activating agents has been evaluated and showed great promise. Polymer-based approaches using polyacryl starch microsphere or mannan resulted in improved effectiveness of antimonial drugs against VL, however to a lesser extent than the liposome-based approach. Cyclodextrin was successfully used for improving the effectiveness of MA by the oral route in a murine model of CL, through the formation of a non-conventional cyclodextrin-antimonial ternary complex that slowly releases 1:1 Sb-meglumine complex on dilution. Gel-forming amphiphilic Sb(V)-guanosine complex is an example of a potential candidate for topical treatment of CL.

8. Expert opinion

From this review, it is clear that drug carrier systems are extremely promising for improving antimonial chemotherapy, the use of liposomes for the targeting of infection sites of VL being the most effective and advanced approach. The high water solubility of pentavalent antimonials makes liposomes the most suitable colloidal carrier for achieving maximal drug-to-carrier ratio. The problem of stability during long-term storage of phospholipid-based formulations can be solved using a freeze-drying process. Although non-ionic surfactants are attractive for the industrial production of vesicular formulations, there is evidence that the resulting formulations may be toxic. SA-containing liposomes, despite promising results, may also be of limited use because of toxicity. Evidence was obtained for the greater effectiveness of liposomes bearing ligand directed to macrophage surface receptors. However, some fundamental questions still have no clear answers, such as how to improve the targeting of infected macrophages with respect to non-infected cells and

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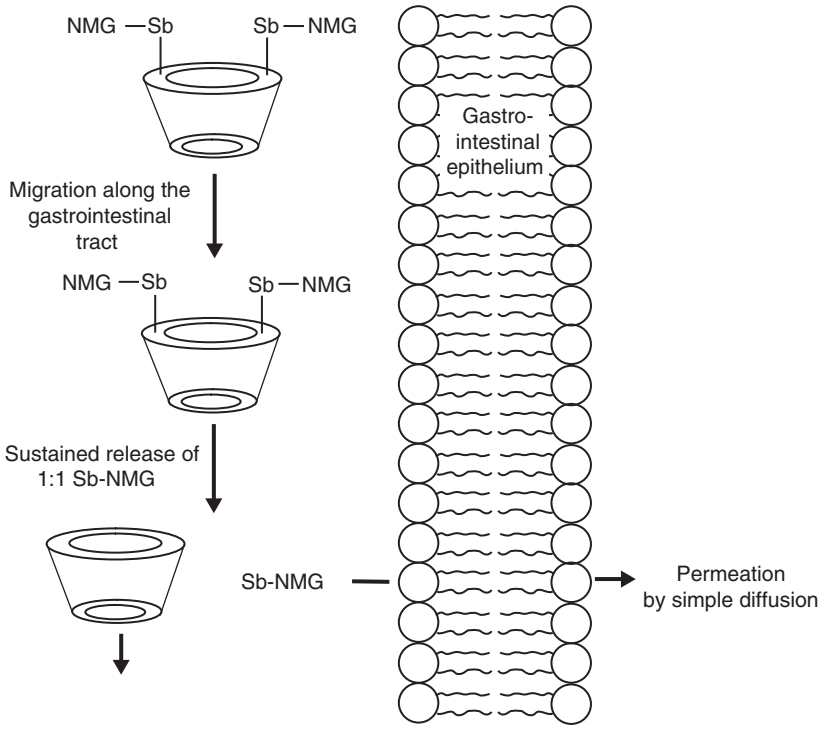

Figure 4. Model proposed for the mechanisms involved in the enhanced absorption of Sb from oral meglumine antimoniate/β-CD composition.

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NMG: *N*-methyl-D-glucamine.

how vesicle characteristics determine, and can be tailored to control, the pharmacokinetics of encapsulated drug.

The achievement of parasitological cure in dogs with VL remains a great challenge. Studies performed in naturally infected dogs suggest that liposome-encapsulated antimonial drug alone cannot lead to complete parasite elimination. However, the approach consisting of the association of the liposomal drug with a macrophage-activating agent, such as Th1-inducing *Leishmania* antigen, deserves further investigation. It is noteworthy that antimony chemotherapy of canine VL is not allowed in the endemic countries where antimonials are the first-line drug for human leishmaniasis, mainly because dogs are the main reservoir of VL and such treatment may increase drug resistance. Thus, a new liposome formulation and dosing regimen, to be applicable to the clinical treatment of infected dogs, should prevent the transmission of parasites to the sandfly without inducing drug resistance. Although this latter point still requires investigation, it is often hypothesized that liposomal therapy, when compared with conventional therapy, may reduce the risk of the appearance of drug resistance by promoting a very high drug concentration at the target starting from the first dose and a shorter treatment course of therapy. Furthermore, in the cases where drug unresponsiveness is caused by upregulation of ABC transporters in host cells [6], liposome delivery may emerge as an effective strategy to circumvent drug resistance.

Liposome-encapsulated antimonial drugs are extremely promising for improving the treatment of VL in humans. From preclinical studies, it is anticipated that liposomes would allow a reduction of applied dose and frequency of dosing. The results of clinical studies with AmBisome also suggest a general tendency that very short-course liposome-based therapy can be at least as effective against VL as the long-course conventional therapies. Finally, reduction of metal-related side effects, enhanced drug effectiveness and improved patient compliance are the main expected benefits. It is often considered that the relatively high cost of phospholipids represents a major obstacle to the development of liposomal formulations. However, it is important to point out that, in the specific case of antimonial formulation, the cost of liposome-based therapy is expected to be less than that of conventional therapy, as a much lower amount of Sb would be used and the cost would be determined mainly by lipids [19]. Furthermore, the short- versus long-course therapy of human VL would reduce considerably the cost related to hospitalization and laboratory monitoring. Ultimately, the decision to continue using pentavalent antimonials as first-line drugs in developing countries and to invest in the development of new liposomal formulations should take into account a recent important international agreement: a preferential price for AmBisome, specifically for developing countries, with a cost reduction of the 50 mg vial from $200 to $20 [14]. Considering this new

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fact and the high effectiveness and low toxicity of AmBisome even in a single high-dose regimen [14], the substitution of pentavalent antimonials by AmBisome seems to be a matter of time. Nevertheless, based on estimated treatment cost, a liposome formulation of antimonial drug may still be able to compete with AmBisome. Assuming that the cost of lipids is ~ $10 per gram (based on the price of lipids in the Lipoid GmbH catalogue) and that of Glucantime is ~ $1.2 per 5 ml vial [99], treatment of a 35 kg out-patient using a single dose of 6.5 mg Sb/kg would cost ~ $15, which is less than the value of $150 estimated for the cost of a single infusion of 10 mg of AmBisome/kg [14]. Indeed, this comparison assumes that liposomal antimony would be as effective as AmBisome in a single-dose therapy of VL, but this still needs to be established.

Another important achievement in the drug delivery of antimonial drugs is the recent report of an orally active formulation of a pentavalent antimonial drug, and so the first experimental evidence that oral bioavailability of these drugs can be improved. However, the potential of this specific oral formulation for application in large animals seems limited, owing to its low water solubility and the modest oral bioavailability of Sb. Further improvements are expected from the association of pentavalent antimonials to other chemical entities with absorption-enhancing capability and taking advantage of our growing knowledge of the chemistry of pentavalent Sb. Indeed, this study stimulates the search for new and even more effective chemical entities for stabilizing the low-molecular-mass 1:1 Sb-meglumine complex and enhancing its intestinal or cutaneous absorption.

Declaration of interest

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