New Formulations and Derivatives of Amphotericin B for Treatment of Leishmaniasis

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Abstract: The clinical treatment of leishmaniasis is based on a limited number of drugs, which are associated with adverse effects and have already induced resistance. Amphotericin B (AmB), a polyene antibiotic produced by Streptomyces sp, is the only anti-leishmanial drug which has not induced clinical resistance since its discovery in 1956. The limiting factor in the use of AmB is its toxic effects, mainly nephrotoxicity. The maximal dose of AmB for human use is 1.5 mg/kg which sometimes is not sufficient for cure. The mode of action of AmB is associated with its toxicity: it selectively binds to parasite membrane ergosterol but also, to a lesser extent, to human cholesterol. Apart from this mechanism, AmB has immunomodulatory effects, some of them are deleterious. Reduction of the toxic effects by using lipid formulations allows the infusion of higher doses of AmB. Unfortunately, these formulations are relatively expensive and therefore out of reach for patients in need, in the endemic areas. All the existing formulations are given parenterally, which has obvious disadvantages; most important is the need for hospitalization or multiple visits in the clinic. The current efforts to improve AmB are directed at the production of AmB aggregates in liquid solutions, encapsulation with lipid components, and solubilization by binding to soluble polymers. The expected improved treatment resulting from use of the new formulations is based on better pharmacokinetics, reduced toxicity originating from slow release, targeting to the infected organ and an altered pattern of immune responses (related to AmB). Of particular importance are the attempts to produce derivatives for oral treatment, which will decrease costs of hospitalization and improve applicability for children and the elderly population.

Keywords: Amphotericin B, AmB derivatives, Leishmaniasis.

INTRODUCTION

It is a common dogma that Amphotericin B (AmB) targets pathogens, which include ergosterol as their main sterol rather than cholesterol, the prevalent sterol in animal cells, and therefore, it is an efficient drug against both leishmanial and fungal infections. However, the treatment of these infections with AmB is complicated and often is not successful. This review attempts to reveal the complications which are associated with AmB treatment and to present new formulations of AmB. In view of the limited scope of the review, we concentrate on new compounds for the treatment of leishmanial infections. However, some AmB derivatives which have not yet been examined against leishmania parasites but depict anti-fungal effect, are also mentioned because of the existence of the common target.

LEISHMANIASIS

Leishmaniasis is the result of infection with intracellular protozoan parasites belonging to the genus *leishmania*. Currently the leishmaniases are prevalent in sub-tropic and tropic areas mostly in developing countries. There are 350 million people at risk, about 600,000 new cases annually, and about 75,000 annual deaths. In addition, HIV has compounded the re-activation of leishmaniasis [1].

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The protozoan parasites that cause leishmaniasis are members of the order Kinetoplastida, family Trypanosomatidae. The organisms are found in two morphologic forms during their life cycle. In humans and other mammalian hosts, they exist within macrophages as round to oval nonflagellated amastigotes, 2 to 3 µm in diameter. In the arthropod vectors (sandflies belonging to the genus *Phlebotomus* or *Lutzomyia*), the parasites exist as elongated flagellated promastigotes, 10 to 15 µm in length and 2 to 3 µm in width.

The clinical patterns are divided into three main categories: cutaneous, mucocutaneous and visceral leishmaniasis.

a. Cutaneous leishmaniasis (CL): The disease begins as a small erythematous papule, which may appear immediately after the bite of the sandfly or 2 to 4 weeks later. The papule slowly enlarges in size (to 2 cm or more) over a period of several weeks and assumes a more dusky violaceous hue. Eventually the lesion becomes crusted in the center. When the crust is removed, a shallow ulcer is found, often with raised and somewhat indurated borders. Small satellite papules may also be found at the periphery of the lesion, and occasionally subcutaneous nodules develop along the course of the proximal lymphatics. After the lesion has been present for 2 months or more, peripheral spread stops, and the ulcerated nodule remains approximately the same size for another 3 to 6 months, or even longer. The lesion then heals, usually leaving a small scar.

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b. Mucocutaneous leishmaniasis (MCL): This disease is a serious and occasionally life-threatening form of leishmaniasis, found mainly in the new world. MCL is characterized by the involvement of both the skin and the upper respiratory tract. MCL begins with a cutaneous lesion that is identical to that of CL. However rather than healing as in CL, the infection extends to the mucosa and eventually to the cartilage of the upper respiratory tract, especially the nose, oral pharynx, and rarely the larynx. Edema and inflammatory changes occur that lead to epistaxis and coryzal symptoms. Eventually there is destruction of the cartilaginous structures in the area, including the nasal septum, the floor of the mouth, and the tonsilar areas. The disease leads to marked disfigurement, and if not arrested, death usually results from superimposed bacterial infection or pharyngeal obstruction leading to acute respiratory failure or malnutrition.

c. Visceral leishmaniasis (Kala-azar, VL): In this disease the parasite establishes itself mainly in the bone marrow, spleen, and liver. VL, if untreated, is often fatal. The incubation period may last from weeks to months; then there is often a subacute febrile onset that may be so severe as to be fatal or so slight that it may be little remarked upon. The next symptom to appear is usually splenomegaly, then pancytopenia, fever, wasting, and serum imbalance of proteins [2, 3].

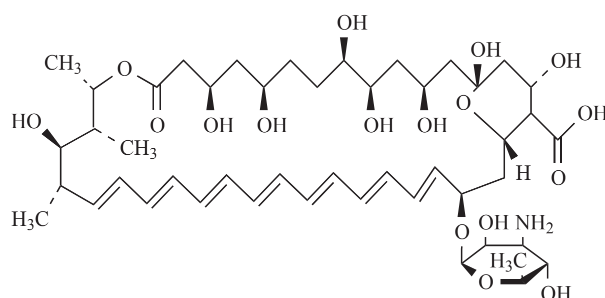

Fig. (1). Amphotericin B.

AMPHOTERICIN B

Amphotericin B (AmB) is one of a large group of polyene macrolide antibiotics produced by *Streptomyces sp.* AmB was first isolated from *S. nodosus* by Gold *et al*. in 1956 [4]. In 1959, Dutcher, *et al.* [5] produced the first patent of amphotericin B production and since then its importance is significantly growing in the treatment of leishmanial and fungal infections. AmB contains a rigid nonpolar heptane unit and a more flexible polyol region fused together in a macrolactone ring. At one end of the molecule there is a mycosamine group and a carboxyl group (Fig. **1**).

The presence of these groups renders the antibiotic zwitterionic in neutral aqueous solutions. AmB is insoluble in water, sparingly soluble in methanol, and highly soluble in dimethyl sulfoxide (DMSO) and dimethylformamide (DMF). The overall length of this molecule is nearly 25 Å which is about half the thickness of a phospholipid bilayer. A space filling model of the head-to-tail x-ray unit cell dimer structure, reveals a polar face with the -OH groups of the polyol regions pointing upwards, and a very apolar face consisting of the polyene region. Capping off the ring structure is a very polar "head group" consisting of the mycosamine sugar and carboxyl group [6]. Chemical modification has shown that suppression of charge on the exocycliccarboxyl group of amphotericin B substantially reduces toxicity. Carmody *et al*. propose a new approach for reducing amphotericin toxicity. They report targeted deletions of the amphN cytochromeP450 gene from the chromosome of the amphotericin-producing bacterium *S. nodosus*. The mutant strains produced amphotericin analogues in which methyl groups replace the exocycliccarboxyl groups. These compounds retained antifungal activity and had reduced hemolytic activity [6a].

MODE OF ACTION OF AmB

a. Selective Binding to Ergosterol

The primary damaging action of AmB is mediated by its binding to sterols incorporated in cellular membranes: ergosterol in the case of fungal and leishmanial cells, and cholesterol in mammalian cells. It has been proposed that the

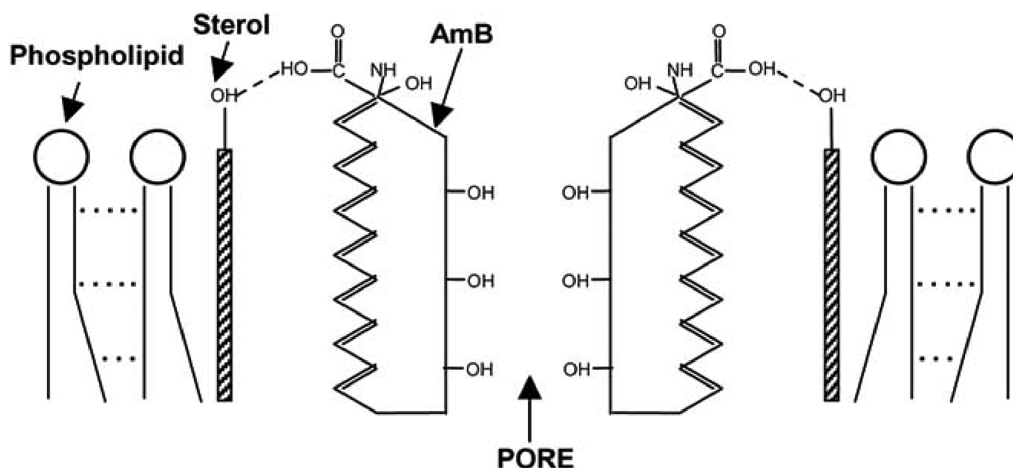

Fig. (2). Schematic representation of the interaction between AmB and sterol in a phospholipid bilayer. The dotted lines between the hydrocarbon chains of the phospholipids represent short-range van der Waals forces. The dashed lines represent hydrogen bonds formed between amphotericin B and sterol molecules.

interaction of AmB with membrane sterols results in the production of aqueous pores consisting of an annulus of eight AmB molecules linked hydrophobically to the membrane sterols (Fig. **2**). This configuration gives rise to a pore in which the polyene hydroxyl residues face inward, leading to altered permeability, leakage of vital cytoplasmic components, and death of the organism [7]. More avid binding of AmB to ergosterol than to cholesterol, and to ergosterol containing membranes than to cholesterol containing membranes is the basis of the selectivity of AmB towards leishmanial and fungal cells [8,9]. There are two kinds of binding of AmB to sterols. First, AmB and sterols with the participation of H_2O, may form a "cage" resulting from hydrogen bonds. These bonds are regulated by proton donor-acceptor forces. The functional groups involved in the hydrogen bonds are the hydroxyl groups of the sterols and the carboxyl group at C-18 of the AmB molecule. This binding is strengthened by participation of the amino group of AmB. Both cholesterol and ergosterol are 3-β-hydroxy sterols, and it can be assumed that their reactions with AmB involving hydrogen bonds are equivalent. The second type of interaction, involving the rigid chain of seven conjugated double bonds of AmB and the whole sterol molecule, is governed by van der Waals forces. Herve and co-workers [10] concluded that the alkyl side chain of ergosterol with the double bond located at C-22 was responsible for the greater sensitivity to AmB of ergosterol containing membranes compared with cholesterol containing membranes. Conformational analysis showed that the overall shape of most ergosterol conformers is flat. In contrast, a flat shape is only one of the possible conformations of cholesterol because its side chain without the double bond at C-22 is more flexible. The flat shape of the ergosterol molecule may facilitate intermolecular contacts with the polyene macrolide [11].

b. Effects on Immune Functions

Apart from the above mechanism of action, AmB appears to have some important immunomodulatory effects. AmB has been shown to cause release of cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) from monocytes and macrophages [12,13]. It has been suggested that these cytokines are also involved in AmB associated acute infusion-related reactions including fever and chills [14]. It was also shown that AmB increases reactive oxygen species production [15], and NO levels [16]. Derivatization of AmB in order to reduce its toxicity or to alter its pharmacokinetics also changes its effect on immune functions and consequently the therapeutic activity of the derivative [17, 18]. We investigated the effect of the water soluble amphotericin B-arabinogalactan (AmB-AG) conjugate (Figs. **3a** and **3b**) on several immune functions. The experiments were of two types: effects of the conjugate on *a*. cytokine release, tumor necrosis factor-α (TNF-α), nitric oxide (NO), and interferon-γ (IFN-γ) release by phagocytic cells, and *b*. cell mediated immune responses. AmB-AG increased TNF-α production by mouse peritoneal macrophages and human monocytes, but had no effect on IFN-γ and NO production. Fungizone, the commercial reference for AmB, also increased TNF-α production but to a lesser extent than AmB-AG. The AG control had no effect on TNF-α production, proving that AmB alone caused the observed increase. AmB-AG and Fungizone were tested for their effect on B and T cell proliferation. Only Fungizone had a slight effect on T-lymphocyte response to concanavalin-A, but both inhibited the stimulation of B-lymphocytes by lipopolysaccharide. However, Fungizone showed a stronger inhibitory effect on B cells. Allocytotoxicity was also inhibited by AmB-AG and more strongly by Fungizone (the effects are summarized in Table **1**). The increased production of TNF-α by cells treated with AmB-AG, and the lower inhibitory effect of AmB-AG on lymphocyte stimulation, and allocytotoxicity, as compared to Fungizone, explains the better therapeutic efficacy of the AmB-polysaccharide conjugate [19]. This assumption is supported by *in vivo* results: treatment of BalbC mice with AmB-AG or AmBisome caused no observable histopathological damage in the kidneys. In contrast, treatment with AmB-DOC (Fungizone) resulted in disruptive changes and apoptosis in renal tubular cells (Figs. **6a** and **6b**). These effects were found to correlate with induction of high levels of IL-1b and TNF-α in kidney lysates. Unlike AmB-AG, AmB-DOC also induced enhanced IL-1b and TNF- α expression in lungs, heart, liver and spleen. The marked elevation of these inflammation-apoptosis-promoting cytokines after treatment with AmB-DOC may mediate its systemic and local renal damage. Treatment with AmB-AG (but not AmBisome) appears to uniquely modulate the in situ expression of IL-1b and enhance secretion of TNF- α in kidneys, effects possibly involved in prevention of apoptosis.

Table 1. The Effects of Fungizone and AmB-AG on Immune Functions

Parameter	Fungizone	AmB-AG
Lymphocyte proliferation	↓↓	↓
Allocytotoxicity	↓↓	↓
Phagocyte reactive oxygen species production	↓	↓
TNF production by leukocytes	↑	↑↑
NO production	=	=
Replication of leishmania parasites	↓	↓↓

↓ Slight decrease.
↓↓ Pronounced decrease.
↑ Slight increase.
↑↑ Pronounced increase.
= No change.

Thus, AmB-related toxicity is associated with induction of IL-1b,TNF-a and apoptosis in various organs. These effects were not observed with AmB-AG, suggesting its potential as a safer formulation for therapy [18]. Overall, the *in vitro* and *in vivo* results suggest that *a*. the anti-leishmanial activity of AmB derivatives is mediated both directly (*via* the specific binding to ergosteriol) and by immunomodulation and *b*. the alleged toxicity of AmB may be related at least partially to deleterious immune responses (see also the next paragraph "Toxicity").

TOXICITY

Despite its proven track record, there has been reluctance to use AmB. The requirement for parenteral administration for long periods of time is inconvenient, frequently

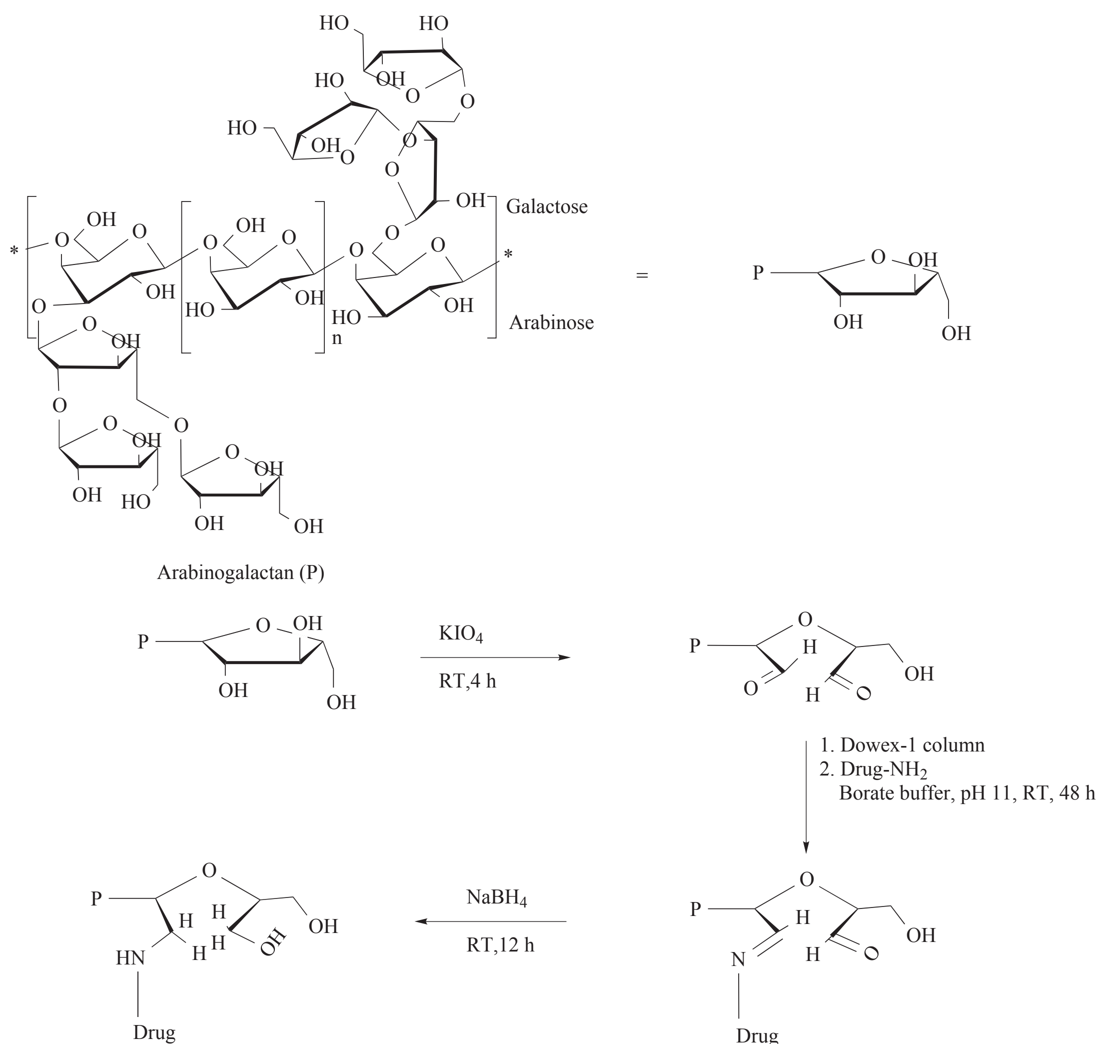

Fig. (3a). Synthesis of AmB- arabinogalactan conjugates.

necessitating hospitalization and prolonged intravenous access. Clinical use of AmB is also limited by the frequent toxic reactions: nephrotoxicity is ultimately the dose limiting factor in many patients, particularly when AmB is used in combination with other potentially nephrotoxic agents (aminoglycosides, cyclosporine, etc.), or in situations in which renal damage is of extreme concern. In addition, acute toxicity manifested by fever, chills, nausea, vomiting, diarrhea, and headache, is common during AmB infusion. This makes the treatment unbearable for some patients [20-22].

PHARMACOKINETICS

The half life of AmB in the serum is relatively long (about 24 h), and the serum concentration is 1.5-3.5 μg/ml (although its spinal concentration is 40 times lower). However, measured blood levels do not correlate with efficacy because the drug is mostly bound to lipoproteins. After 24 hours, most of the drug is accumulated in various organs, especially the liver, but serum concentrations may be detected for up to 7 weeks because of the release from cell membranes [23]. Only 5% of the drug is metabolized directly by the kidneys; the remaining dose is excreted by the bile and feces [24]. In agreement with the assumption that toxic reactions to AmB increase with more rapid infusion, a longer time of infusion to human patients reduced the toxicity, especially renal toxicity [23, 25]. Thus, any formulation that prolongs the release of the drug would reduce its toxicity (see "AmB formulations which are approved for human use"). For example liposomal AmB produces higher plasma concentrations, decreases renal and fecal clearance and reduces toxicity [26, 27]. AmB

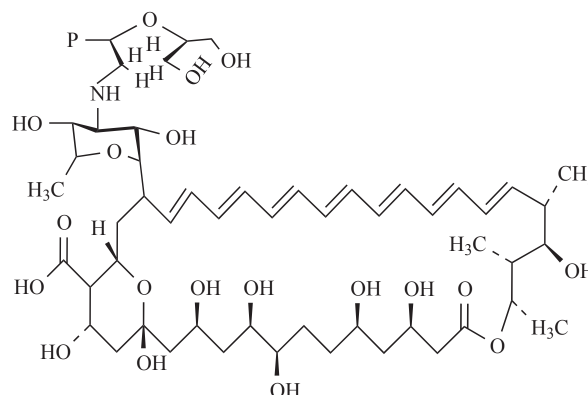

Fig. (3b). Binding of the AmB to the polysaccharide by reductive amination *via* the AmB amine side group.

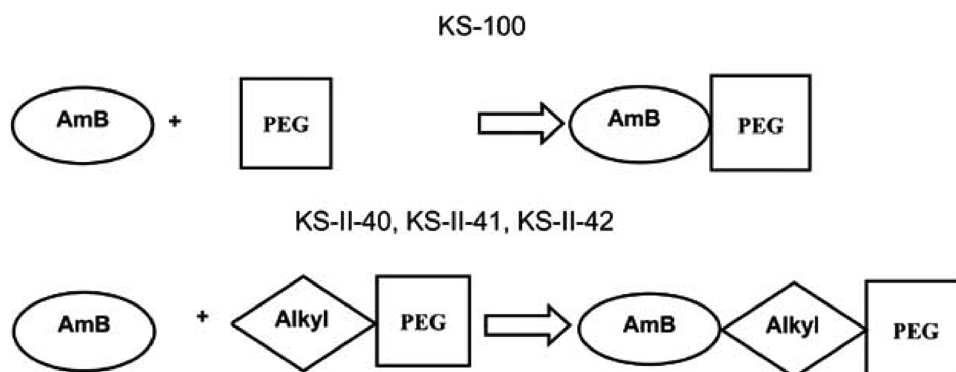

Fig. (4). Schematic description of experimental design.

concentrations achieved in the blood after administration of Amphotericin B Lipid Complex (ABLC, Abelcet) are lower compared to those achieved with AmB deoxycholate. On the other hand, ABLC produces higher concentrations in liver, spleen, and lungs. The renal concentration is similar for the two formulations. Importantly, when ABLC is administered at higher doses, its concentration in the kidneys increases only slightly and that in the plasma remains the same [28, 29].

RESISTANCE TO AmB

Despite more than 30 years of clinical use, resistance to polyene antibiotics such as amphotericin B is not described at clinical level. AmB resistance was induced only *in vitro* and was suggested to be associated with gene amplification [30]. In another case AmB resistance was associated to the absence of 24-alkylated sterols such as ergosterol in the membrane of a leishmania mutant [31]. However, in a precise investigation of a resistant *L. donovani*, it was demonstrated that the S-adenosyl-l-methionine:C-24-Delta-sterol-methyltransferase (SCMT or ERG6) was not functional or not expressed in the AmB-resistant parasites. This could explain why the 24-alkylated sterols have been shown previously to be absent in membranes of the AmB resistant *Leishmania* [32].

AmB FORMULATIONS APPROVED FOR HUMAN USE

The first commercial AmB formulation was Fungizone, a colloidal dispersion with sodium deoxycholate. It has been in clinical use since 1958 but it is highly toxic: the maximal dose, 1.5 mg/kg/day is sometimes not sufficient for cure.

Reduction of the toxic effects was accomplished by formulation of AmB with lipids that allow the infusion of higher doses of AmB. Several AmB liposomal preparations have been developed, including Amphotec (Amphocil), Abelcet, and AmBisome [33-35]. Table **2** summarizes the properties of the AmB formulations. These lipid formulations present lower toxicity as compared to Fungizone and can be administered to patients at doses up to 5 mg/kg/day. It is hypothesized that once AmB is

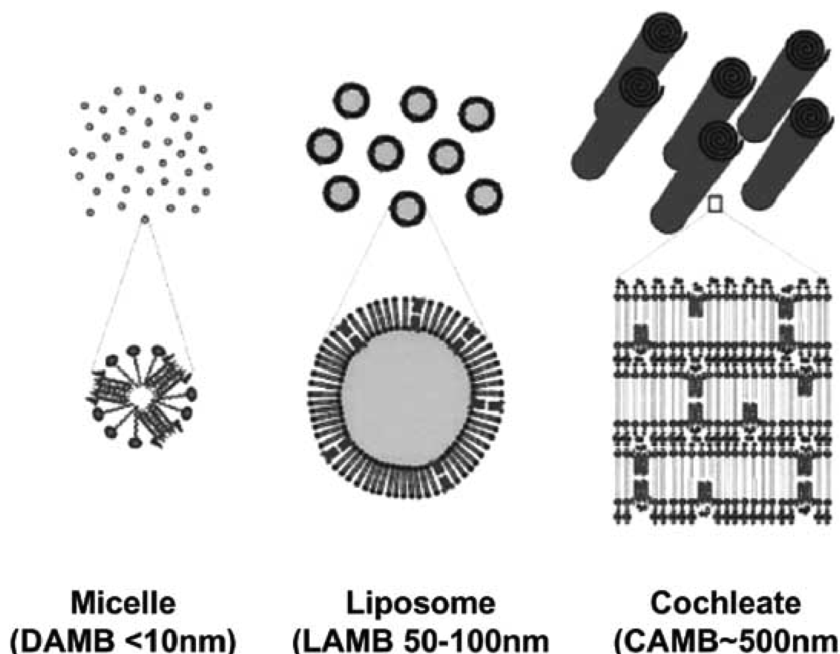

Fig. (5). Schematic representation of physical states of AmB delivery suspensions, including dispersed detergent micelles, ordered liposomal vesicles, and rolled crystalline [59].

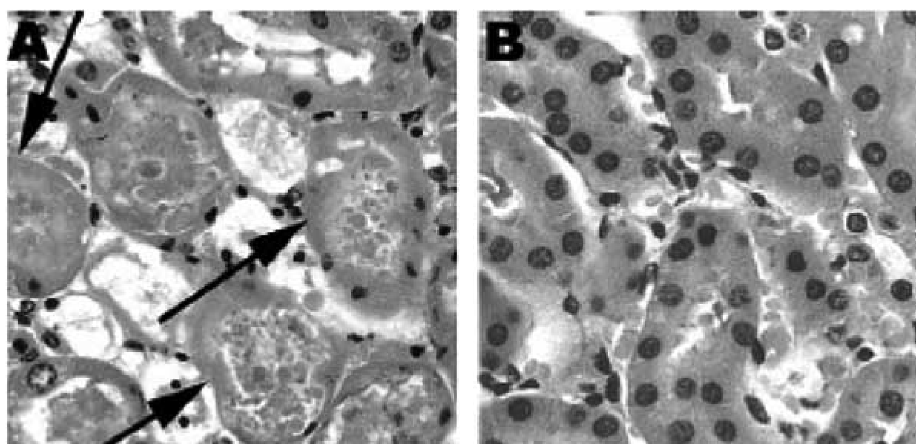

Fig. (6a). Hematoxylin and Eosin (H and E) staining of mouse kidney sections after treatment with different AmB formulations. Histological findings are shown in black and white, and represent the original H&E staining. The mice were iv injected with 4 mg/kg of (A) AmB-DOC and (B) AmB-AG conjugate, and the kidneys were excised 4 h post-injection. In (A), arrows point to necrotic tubular epithelium. No damage to the renal epithelium was noted in the AmB-AG treated mice (B, arrows). Magnification, 250 [18].

incorporated into liposomes, it may participate in a selective transfer mechanism, which enables its transfer from the "donor" liposome to the ergosterol-containing "target" in the cell membrane aided by the parasite and/or host phospholipases [36].

PARTICULATE FORMULATIONS

AmB is lipophylic and therefore is not soluble in liquid solutions. However, AmB can form different aggregates in physiological media, which are stabilized by hydrophobic and electrostatic forces and affect the interaction with sterols in biological membranes [37]. Sanchez-Brunete *et al.* examined formulations of different aggregate size in hamsters and found that transformation of water soluble AmB into larger insoluble AmB suspended with deoxycholate, reduced toxicity and improved anti-leishmanial (*L. infantum*) efficacy. In contrast, separation

Table 2. Chemical and Physical Properties of Commercial AmB Formulations

	Lipid configuration	Size (nm)	Lipid component
Fungizone	Micelle	<25	Sodium deoxycholate
Abelcet	Disk-like	125	Cholesteryl sulfate
Amphotec	Ribbon-like	500-5000	Dymirystoyl phosphatidylcholine dimyristoyl phosphatidylglycerol
AmBisome	Unilamellar vesicle	90	Hydrogenated phosphatidylcholine Cholesterol distearoylphosphatidyl-glycerol
Bioral	Unilamellar vesicle	407	Phosphatidylserine

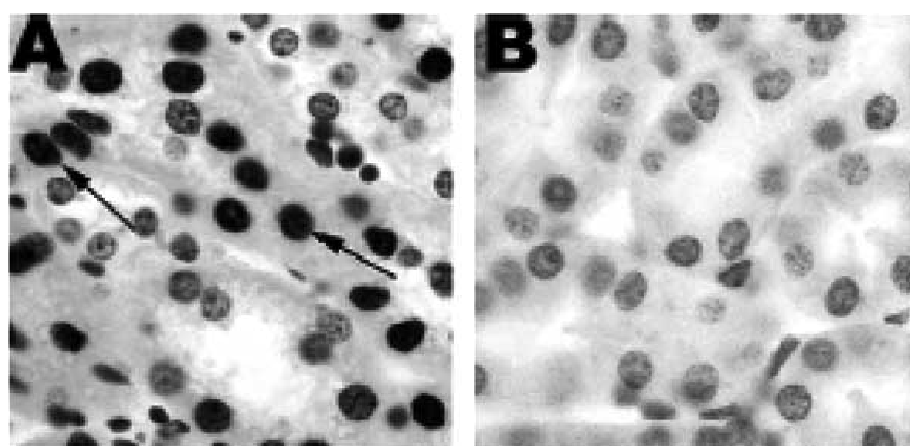

Fig. (6b). Apoptotic cells detected by the TUNEL method in mouse kidney sections. The mice were iv injected with 4 mg/kg of AmB-DOC (A) and AmB-AG conjugate (B), and the kidneys were excised 4 h post-injection. No apoptotic cells are demonstrated after treatment with AmB-AG in panel (B), in contrast with the numerous TUNEL-stained apoptotic nuclei found after treatment with AmB-DOC (A).Arrows point to stained nucleus in apoptotic cells. The dark-black colour represents the original brown-dark staining of nuclei in apoptotic cells. Magnification, 400 [18].

into soluble monomers in γ-cyclodextrin enhanced acute toxicity [37].

In general, all particulate formulations both commercial and experimental are more efficient than the native AmB. The improved treatment of leishmaniasis with particulate preparations in both animal models and humans, can be attributed to modified toxicity, release, pharmacokinetics and immune responses [17,38,39]. A particulate or aggregated AmB may be also considered "targetable" to the macrophage because of their phagocytic nature. These concepts are demonstrated by Cheron *et al.* [40]: heat-induced reformulation of AmB-deoxycholate favors drug uptake by the macrophage-like murine cell line J774. The *in vitro* toxicity of their "superaggregates" was also reduced in comparison with the untreated AmB-DOC.

AmB ENCAPSULATED WITH LIPID COMPONENTS

Of particular interest is Ambisome; a commercial AmB-liposomal formulation successfully used in humans infected with all variations of the diseases. Because of its excellent safety profile, a large dose (7.5 mg/kg) of Ambisome was given to each of 203 patients with VL. 183 patients (90%) had obtained final cure 6 months after treatment [41]. However, Ambisomes' shelf life is limited and it is too expensive for use in the endemic areas where it is mostly needed. Various attempts to produce substitutes for Ambisome have been reported. AmB nanoparticles (AmB-NP) were prepared by a solvent displacement method: poly(ε-caprolactone) and amphotericin B were dissolved in a mixture of organic solvents (methanol/acetone; 1:2 v/v) and acidified with 0.1 M HCl. This organic phase was heated at 50°C and then poured into distilled water containing poloxamer 188, under moderate magnetic stirring. The organic solvents were eliminated by evaporation under vacuum and the nanoparticles (AmB-NP) concentrated to 10 ml. The nanoparticles were relatively small (280nm) and less toxic than AmB *in vitro* and *in vivo*. However, their biological activity as measured in mice infected by Candida albicans, was inferior to that of AmB-deoxycholate (Fungizone equivalent) [42]. Thus, reduced toxicity and particle size do not necessarily predict the *in vivo* effect. Recently, a delivery system, Ionic Amphiphilic Biovector (ABV), comprised of anionic lipids (dipalmitoyl phosphatidyl glycerol) included in a cationic cross-linked polysaccharide matrix was used as a reservoir for AmB. Consequently sub-microscopical, stable AmB containing particles were formed. Two ABV formulations exhibited an *in vitro* and *in vivo* efficacy similar to AmBisome, in *L. donovani*-infected Balb/c mice. The higher stability of these ABV formulations indicates their potential for further development and applications [43]. Another attempt to produce a less toxic AmB delivery system was ABCV, a preparation of AmB in a cholesterol hemisuccinate vesicle [44]. AmB and cholesterol hemisuccinate were mixed in 1:2 molar ratio in methanol. Lactose, a cryopreservant, was added to a final concentration of 10% in the ABCV. The dry film obtained on rotary evaporation was hydrated with 10 mM Tris-HCL, 0.1 mM EDTA, pH 7.4, sonicated and French pressed. It was then extruded through a 1.2 µm filter. The final AmB concentration of the product was 6mg/ml. The mean particle size was 252 nm. This preparation reduced the toxicity of AmB (compared to Fungizone) in Balb/c mice. The reduced toxicity was attributed to the accumulation of AmB in the liver and spleen.

Table 3. Derivatives of AmB Classified According to the Number of PEG Residues

PEG OH-(CH_2-CH_2-O)n-H	Derivative
n=7	KS-100
n=7	KS-ll-40
n=16	KS-ll-41
n=45	KS-ll-42

Microencapsulation is a drug delivery technique that is used to increase bioavailability, enhance drug stability, reduce adverse or toxic effects, or extend drug release. The clinical use of some effective therapeutic substances, such as paclitaxel or AmB, has been limited because of their restrictive physicochemical properties which have required frequent administration, and their side effects. It is possible that these substances may become more widely used in a clinical setting, if appropriate microencapsulation techniques can be designed to overcome their poor oral bioavailability, intrinsic toxicity, and insolubility. The objective of microparticulate delivery systems is to enhance transport of drugs through biological membranes, and to control and extend the release of the active ingredient from a particle without modification of its activity in the body. A vast number of microparticulate systems have been devoted to the development, characterization, and potential applications of specific microparticulate and nanoparticulate delivery systems. [45,46]. These new drug delivery systems were also applied for AmB. Sanchez-Brunete *et al.* [47] treated hamsters infected with *L. infantum* by i.v. injections of AmB containing lyposphares. AMB microspheres were prepared by spray drying. AMB was dispersed in an aqueous dissolution containing sodium deoxycholate, dibasic sodium phosphate, and monobasic sodium phosphate. The resulting dispersion was subjected to moderate stirring with until a homogeneous suspension was achieved. A 20% serum albumin solution was added, and the final mixture was spray dried. Microspheres were collected and heated at 60°C for 1 h. Before drug injection, the AMB microspheres were dispersed in a 5% glucose-water solution. The mean particle size was 1µm. The higher doses of AmB in microspheres, 10, 20, and 40 mg/kg, reduced parasite levels in the liver and spleen by more than 99%. A pharmacokinetic study depicted a significant accumulation of AMB in the spleen and liver. These results correlate with those of Townsend *et al.* [48] who injected radioactive Ambisome to rats. This is obviously an advantage in treatment of VL because the parasites accumulate mostly in the liver and spleen in visceral leishmaniasis.

SOLUBILIZATION ATTEMPTS

There were a few attempts to conjugate side chains to AmB in order to solubilize it. We attempted several modes of derivatization including conjugation to *a*. alkyl-polyethylenglycol (PEG) [unpublished data] and *b*. arabinogalactan.

a. The derivatives were synthesized by conjugating the primary amine in the nucosamine- moiety of AmB to an amphiphilic residue composed of different PEG bound covalently to alkyl chains (Scheme, Table **2**). AmB was directly connected to a hexyl chain which itself was bound to Methoxy-PEG- (350) (KS-II-40), Methoxy-PEG- (750) (KS-II-41), Methoxy-PEG- (2000) (KS-II-42). AmB conjugated to Methoxy-PEG- (350) served as a control for the effect of PEG alone on AmB properties. All the compounds were soluble in both organic and aqueous solutions. *In vitro* examinations against *L. major* promastigotes and amastigotes, J 774 mouse monocytes, and H1 melanoma revealed a therapoietic index (TI) of about 100. However, the TI of fungizone was approximately 500. KS-100 was the most potent derivative in both *in vitro* and *in vivo* examinations. All the derivatives reduced the area of the lesion in the mouse model (Balb/c mice infected with *L. major*), but only fungizone arrested the development of the lesion.

b. We [49] synthesized a new stable, highly water-soluble, nontoxic polysaccharide conjugate of amphotericin B (AmB). AmB was conjugated by a Schiff-base reaction with oxidized arabinogalactan (AG). The conjugation reaction (Fig. **3**) is inexpensive; it is carried out in aqueous medium at room temperature without organic solvents. A high yield of active AmB was obtained in the conjugates (30% w/w AmB content), which were highly water soluble (>100 mg/ml) and could be appropriately formulated for injection. These conjugates showed low MIC values against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (0.1-0.2 μg/ml). The reduced AmB conjugate which was synthesized at pH 11 for 48h at 37^0C, was non hemolytic and much safer than conventional micellar AmB-deoxycholate. It was the least toxic formulation among those tested in mice (maximal tolerated dose 50 mg/kg), and histopathology indicated no damage to liver and kidneys. This conjugate, similar to AmBisome, was more effective than the AmB-deoxycholate in prolonging survival in three different animal models (murine candidiasis, murine cryptococcosis and murine aspergillosis). Furthermore, it was more effective than both the liposomal and the deoxycholate formulations in eradicating yeast cells from target organs. The AmG-AG conjugates were also found to be highly effective in treating leishmaniasis in a mouse model.

KY62 is a synthetic, water-soluble polyene with structural resemblance to AmB. Instead of the hydroxyl group in the carboxyl, it has a residue of $NH(CH_2CH_2O)_6CH_3$. KY62 is produced by suspension of AmB in dimethylacetamide and treatment with triethylamine, diphenylphosphorylazide and methoxyethylamine, in a nitrogen atmosphere. After 115h, the mixture is poured into ethyl ether and the crude product solubilized and precipitated 4 times by CH_2OH/ethyl ether systems. The resulting product is further purified by silica gel chromatography to give R_f 0.58 [50]. This drug was administered by i.p. injections to Balb/c mice in high doses of up to 30 mg/kg with no noticeable toxicity. The activity of KY62 against *Leishmania* promastigotes *in vitro*, in experimental cutaneous leishmaniasis caused by *L. amazonensis*, and in experimental visceral leishmaniasis caused by *L. donovani* was comparable to injectable doses (1-5 mg/kg) of native AmB [51].

ORAL TREATMENT

Oral treatment is preferable to other methods of drug delivery because of the possibility to shorten or avoid hospitalization, which is associated with the other modes of administration of AmB containing compounds. Recently, much attention has been devoted to lipid-based formulations with emphasis on self-emulsifying drug delivery systems (SEDDS) to improve oral bioavailability of lipophilic drugs [52]. The liquid preparations are extremely important for two large populations - children and elderly - who both suffer from difficulties in swallowing solid dosage forms. The oral availability of a drug depends on its ability to be soluble in both aqueous and lypophylic medium. This way it would be soluble in the aqueous niches of the gastrointestinal system and later penetrate through the membranes to reach its target organs. One possibility of improving the bioavailability of oral preparations is microencapsulation of the drugs. A correlation exists between particle size and the oral bioavailability: best bioavailability is obtained for formulations forming nanodispersions of less than 100 nm. It is generally considered that the efficiency of uptake of smaller particles by the intestinal tissues is higher compared to microparticles [52]. However, the reason for good bioavailability could also depend on the ability of the drug to adhere to the intestinal wall and to cause reversible local damage of microvillae, which increase permeability. These effects may depend on the nature of surfactants in the particle rather than on particle size [54].

Preliminary steps were taken to enable the use of AmB in oral delivery. These include attempts to solubilize AmB and to increase bioavailability by mixing the drug with micelles composed of phospholipids and encapsulation of the drug in nanosuspensions. Improved GI absorption of poorly absorbable drugs can be achieved by increasing the dissolution rate of the drug in the presence of bile acids. Within the gastrointestinal tract (GI), bile salts behave as biological detergents that, when mixed with phospholipids, form thermodynamically stable mixed micelles. Numerous studies have reported enhanced absorption of poorly absorbable drugs when administered as mixed micellar solutions. In addition, incorporation of AmB into mixed micelles containing bile acids and phospholipids, resulted in increased intestinal permeability and subsequent GI absorption in a rat intestinal-perfusion system [54]. Risovic *et al.* examined Fungizone, Abelcet, AmB-lipid complex and AmB in Peceol or in Iintralipid. The lipid complex consisted of AmB complexed with two nontoxic phospholipids, L-α-dimyristoyl phosphatidylcholine and L-α-dimyristoyl phosphatidylglycerol, in a 1:1 drug-to-lipid molar ratio. Peceol was chosen for the SEDDS formulation because of the ability of this combination to solubilize AmB at high concentrations while providing an oral delivery system with rapid self-emulsifying properties. Oral administration of both Intralipid-AmB and Peceol-amB increased AmB availability in the plasma and reduced renal toxicity. It was suggested

that Peceol elevates the gastrointestinal absorption of Am B by increasing the amount of drug that is transported through the mesenteric lymph duct. This is based on measurements of AmB that was transported through the mesenteric lymph nodes [56].

Kayser *et al*. [57] employed a nanosuspension technique to produce AmB nano-particles for oral administration. Nanosuspensions of AmB were produced by a high pressure homogenisation technique using a Micron LAB 40 homogeniser. AmB was suspended at a concentration of 0.4% (m/m) in an aqueous solution of Tween 80 (0.5% m/m), Pluronic F68 (0.25% m/m), and sodium cholate (0.05% m/m). The pre-suspensions were dispersed using an Ultra Turrax for 5 min at 9500 rpm. The coarse pre-suspension was homogenised at 150 and 500 bar for two cycles each, then at 1500 bar for 15 cycles. The size reduction process resulted in a suspension in the range of 1μm particles (it would be more appropriate to call the product "microsuspensions"). Balb/c mice were infected by *L. donovani*. Treatment began on Day 7 post infection. The compounds were given orally by feeding at a dose level of 5 mg/kg for 4 or 5 consecutive days. Mice were sacrificed on day 14 post infection and the number of amastigotes in liver touch preparations was counted. The AmB-nanosuspensions reduced parasite load by 29%. There was no parasite reduction by oral administration of Ambisome or Fungizone. Interestingly, man and rats absorb the drug much less readily than mice [58]. Therefore, the rat might be a better model for absorbtion of AmB derivatives. However, the results of Kayser *et al*. justify the use of a mouse model.

The most striking results concerning oral treatment with AmB containing formulations were performed with AmB-cochleates [59]. AmB cochleate (CAMB) is a lipid-based delivery vehicle that has an advantage over existing formulations due to the stability of cochleates and their resistance to degradation in the gastrointestinal tract. Thus, cochleate preparations have the potential to deliver AmB orally. Cochleates are stable phospholipid-calcium precipitates comprised mainly of phosphatidylserine. They have a defined multilayered structure consisting of a continuous, solid, lipid bilayer sheet rolled up in a spiral, with no internal aqueous space (Fig. **5**). Cochleates have been used to deliver protein, peptide, and DNA for vaccine and gene therapy applications and have been used as a drug delivery system. CAMB were prepared using an aqueous/aqueous hydrogel binary system. AmB in methanol was added to L-α-phosphatidylserine in chloroform at a molar ratio of 10:1. The mixture was dried to a mixed drug-lipid film using a rotary evaporator. The film was hydrated with deionized water and the crude AmB-lipid suspension was sonicated to form unilamellar vesicles. The liposome suspension was mixed with 40% dextran (molecular weight, ~500,000) in a suspension of 3/1 dextran/liposome. This mixture was injected into 15% polyethylene glycol 8000 with stirring at 800 to 1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach a final concentration of 1 mM, and stirring was continued for 1 h. A washing buffer was added to the suspension at a ratio of 1:1 and the suspension vortexed and centrifuged. The sample was resuspended in washing buffer and recentrifuged. The final pellet was reconstituted with the same buffer. Laser light scattering indicated that the CAMB mean diameter was 407 nm. CAMB have been shown to be highly protective in a mouse candidiasis model following parenteral administration. Because of the hydrophobic nature of AmB molecules, it was hypothesized that AmB would be localized in the rigid lipid bilayers of the cochleates. This unique association should protect AmB from degradation when exposed to harsh environmental conditions or enzymes. CAMB should be an ideal system to deliver AmB orally. Biodistribution studies of CAMB administered orally in a mouse model showed that cochleates delivered therapeutic levels of AmB to target organs. Oral treatment of Balb/c mice infected by *C. albicans* reduced fungal tissue burden in a dose-dependent manner, comparable to the results of i.p. injection of 2mg/kg/day of Fungizone [59]. The CAMB is marketed commercially (**Bioral™ Amphotericin B).**

FINANCIAL CONSIDERATIONS OF TREATMENT

The estimated cost of treatment may be evaluated to a certain degree of accuracy. It depends not only on the drug of choice but also on the mode of delivery (e.g. *per os* versus intravenous infusion) and the location of the clinic/hospital (e.g. hospitalization is much more expensive in Europe than in India).

An axample of the estimated costs in India, for selected regimens of treatment of visceral leishmaniasis, for a 25-kg adult include: amphotericin B, 30-day treatment (drug = $49, total = $417); AmBisome, 2 mg/kg/day for five days (drug = $800, total = $872); and Abelcet, 2 mg/kg/day for five 5 days (drug = $875, total = $947). The total cost includes all expenses. These regimens cure about 90% of the patients [60].

Unfortunately, a comparison of the expected counter price of experimental drugs to the price of a commercial drug such as AmBisome (which is considered to be "too expensive for use in endemic areas") is impossible: the price cannot be compared because the experimental drugs are not produced in industrial processes and are not available to the public. In addition, there is no price for the new drugs that takes into account commercial considerations (development, marketing, marginal profit, etc.). Therefore, it is premature to declare any experimental drug as a "less expensive compound".

REFERENCES

[1] WHO information by topics or disease: http://www.who.int/emc/diseases/leish/index.html. 2003.

[2] Hepburn, N.C. *J. Postgrad. Med*., **2003**, *49*, 50-54.

[3] Herwaldt, B.L. *Lancet*, **1999**, *354*,1191-1199.

[4] http://www.thedrugmonitor.com/DCAB.html

[5] Butcher, J.D. *Dis. Chest*, **1968**, *54*(Suppl. 1), 296-298.

[6] Harstel, S.C.; Hatch, C.; Ayenew, A. *J. Liposome Res*., **1993**, *3*, 377-381.

[6a] Carmody, M.; Murphy, B.; Byrne, B.; Power, P.; Rai, D.; Rawlings, B.; Caffrey, P. *J. Biol. Chem*., **2005**, *280*, 34420-34426.

[7] Ghannoum, M.A.; Rice, L.B. *Clin. Microbiol. Rev*., **1999**, *12*, 501-517.

[8] Vertut-Croquin, A.; Bolard, J.; Chabbert, M.; Gary-Bobo, C. *Biochemistry*, **1983**, *22*, 2939-2944.

[9] Gruda, I.; Nadeau, P.; Brajtburg, J.; Medoff, G. *Biochim. Biophys. Acta*, **1980**, *602*, 260-268.

[10] Herve, M.; Debouzy, J.C.; Borowski, E.; Cybulska, B.; Gary-Bobo, C.M. *Biochim. Biophys. Acta,* **1989**, *980*, 261-272.

[11] Brajtburg, J.; Powderly, W.G.; Kobayashi, G.S.; Medoff, G. *Antimicrob. Agents Chemother.*, **1990**, *34*,183-188.

[12] Vonk, A.G.; Netea, M.G.; van der Meer, J.W.; Kullberg, B.J. *J. Infect. Dis.*, **1999**, *180*,1408-1409.
[13] Rogers, P.D.; Jenkins, J.K.; Chapman, S.W.; Ndebele, K.; Chapman, B.A.; Cleary, J.D. *J. Infect. Dis.*, **1998**, *178*, 1726-1733.
[14] Chia, J.K.S.; Pollack, M. *J. Infect. Dis.*, **1989**,*159*, 113-116.
[15] Marzzullo, L.; Souza, L.C.; Campa, A. *Gen. Pharmacol.*, **1997**, *28*, 203-207.
[16] Mozaffarian, N.; Berman, J.W.; Casadevall, A. *Antimicrob. Agents Chemother.*, **1997**, *41*, 1825-1829.
[17] Ehrenfreund-Kleinman, T.; Domb, A,; Jaffe, C.L.; Nasereddin, A.; Leshem, B.; Golenser, J. *J. Parasitol*., **2005**, *91*, 158-163.
[18] Falk, R.; Hacham, M.; Nyska, A.; Foley, J.F.; Domb, A.J.; Polacheck, I. *J. Antimicrob. Chemother.,* **2005**, *55*, 713-720.
[19] Golenser, J.; Frankenburg, S.; Ehrenfreund, T.; Domb, A. *J. Antimicrob. Agents Chemother*., **1999**, *43*, 2209-2214.
[20] Sabra, R.; Branch, R.A. *Drug Saf.*, **1990**, *28*, 94-108.
[21] Gallis, H.A.; Drew , R.H.; Pickard, W.W. *Rev. Infect. Dis.*, **1990**, *12*, 308-329.
[22] Chabot, G.G.; Pazdur, R.; Valeriote, F.A.; Baker, L.H. *J. Pharm. Sci.,* **1989**, *78*, 307-310.
[23] Fluckiger, U. *Swiss Med. Wkly*., **2002**, *132*, 431-432.
[24] Bellmann, R.; Egger, P.; Wiedermann, C.J. *Clin. Infect. Dis.*, **2003**, *36,* 1500-1501.
[25] Peleg, A.Y.; Woods, M.L. *J. Antimicrob. Chemother*., **2004**, *54*, 803-808.
[26] Bekersky, I.; Fielding, R.M.; Dressler, D.E.; Lee, J.W.; Buell, D.N.; Walsh, T.J. *Antimicrob. Agents Chemother*., **2002**, *46*, 834-840.
[27] Bakker-Woudenberg, I.A.; Schiffelers, R.M.; Storm, G.; Becker, M.J.; Guo, L. *Methods Enzymol*., **2005**, *391*, 228-260.
[28] Kline, S.; Larsen, T.A.; Fieber, L.; Fishbach, R.; Greenwood, M.; Harris, R.; Kline, M.W.; Tennican, P.O.; Janoff, E.N. *Clin. Infect. Dis*., **1995**, *21*, 1154-1158.
[29] Goldsmith, D.R.; Perry, C.M. *Drugs*, **2004**, *64*, 1905-1911.
[30] Singh, A.K.; Papadopoulou, B.; Ouellette, M. *Exp. Parasitol.*, **2001**, *99*, 141-147.
[31] Mbongo, N.; Loiseau, P.M.; Billion, M.A.; Robert-Gero, M. *Antimicrob. Agents Chemother*., **1998**, *42*, 352-357.
[32] Pourshafie, M.; Morand, S.; Virion, A.; Rakotomanga, M.; Dupuy, C.; Loiseau, P.M. *Antimicrob. Agents Chemother*., **2004**, *48*, 2409-2414.
[33] Hann, I.M.; Prentice,H.G. *Int. J. Antimicrob. Agents*, **2001**, *17,* 161-169.
[34] Andres, E.; Tiphine, M.; Letscher-Bru, V.; Herbrecht, R. *Rev. Med. Interne*., **2001**, *22*,141-150.
[35] Arikan, S.; Rex, J.H. *Curr. Pharm. Des.*, **2001**, *7*, 393-415.
[36] Juliano, R.L.; Grant, C.W.; Barber, K.R.; Kalp, M.A. *Mol. Pharmacol.,* **1987***, 31,* 1-11.
[37] Sanchez-Brunete, J.A.; Dea, M.A.; Rama, S.; Bolas, F.; Alunda, J.M.; Torrado-Santiago, S.; Torrado, J.J. *J. Drug Target*, **2004**, *12*, 453-460.
[38] Falk, R.; Grunwald, J.; Hoffman, A.; Domb, A.J.; Polacheck, I. *Antimicrob. Agents Chemother*., **2004**, *48*, 3606-3609.
[39] Petit, C.; Yardley, V.; Gaboriau, F.; Bolard, J.; Croft, S.L. *Antimicrob. Agents Chemother*., **1999**, *43*, 390-392.
[40] Cheron, M.; Petit, C.; Bolard, J.; Gaboriau, F. *J. Antimicrob. Chemother*., **2003**, *52*, 904-910.
[41] Sundar, S.; Jha, T.K.; Thakur, C.P.; Mishra, M.; Singh, V.P.; Buffels, R. *Clin. Infect. Dis.,* **2003**, *37*, 800-804.
[42] Espuelas, M.S.; Legrand, P.; Campanero, M.A.; Appel, M.; Cheron, M.; Gamazo, C.; Barratt, G.; Irache, J.M. *J. Antimicrob. Chemother*., **2003**, *52*, 419-427.
[43] Loiseau, P.M.; Imbertie, L.; Bories, C.; Betbeder, D.; De Miguel, I. *Antimicrob. Agents Chemother*., **2002**, *46*, 1597-1601.
[44] Saxena,S.; Ghosh, P.C. *Pharm Res*., **2000**, *17,* 1236-1242.
[45] Bernstein, H.; Cohen, S. *Microparticulate systems for the delivery of proteins and vaccines*. Marcel Dekker: New York, **1996**.
[46] Benita, S. *Microencapsulation: Methods and industrial application*. Marcel Dekker: New York, **1996**.
[47] Sanchez-Brunete, J.A.; Dea, M.A.; Rama, S.; Bolas, F.; Alunda, J.M.; Raposo, R.; Mendez, M.T.; Torrado-Santiago, S.; *Antimicrob. Agents Chemother.,* **2004** ,*48*, 3246-3252.
[48] Townsend, R.W.; Zutshi, A.; Bekersky, I. *Drug Metab. Dispos.*, **2001**, *29*, 681-685.
[49] Ehrenfreund, T.; Azzam, T.; Golenser, J.; Domb, A.J. *Biomaterials*, **2002**, *23*, 1327- 1335.
[50] Yamashita, K.; Janout, V.; Bernard, E.M.; Armstrong, D.; Regen, S.L. *J. Am. Chem. Soc*., **1995**, *117*, 6249-6253.
[51] Al-Abdely ,H.M; Graybill, J.R.; Bocanegra, R.; Najvar, L.; Montalbo, E.; Regen, S.L.; Melby, P.C. *Antimicrob. Agents Chemother*., **1998**, *42*, 2542-2548.
[52] Kommuru, T.R.; Gurley, B.; Khan, M.A.; Reddy, I.K. *Int. J. Pharm.,* **2001**, *212*, 233-246.
[53] Bekerman, T.; Golenser, J.; Domb, A. *J. Pharm. Sci*., **2004** ,*93*, 1264-1270.
[54] Andrysek, T. *Mol. Immunol.,* **2003**, *39*, 1061-1065.
[55] Risovic, V.; Boyd, M.; Choo, E.; Wasan, K.M. *Antimicrob. Agents Chemother*., **2003**, *47,* 3339-3342.
[56] Risovic, V.; Sachs-Barrable, K.; Boyd, M.; Wasan, K.M. *Drug. Dev. Ind. Pharm*., **2004**, *30*, 767-774.
[57] Kayser, O.; Olbrich, C.; Yardley, V.; Kiderlen, A.F.; Croft, S.L. *Int. J. Pharm*., **2003**, *254*, 73-75.
[58] Robbie, G.; Wu, T.C.; Chiou, W.L. *Pharm. Res.*, **1999**, *16*, 455-458.
[59] Santangelo, R.; Paderu, P.; Delmas G.; Chen, Z.W.; Mannino, R.; Zarif, L.; Perlin, D.S. *Antimicrob. Agents. Chemother*., 2000, *44*, 2356-2360.
[60] Murray, H.W. *Am. J. Trop. Med. Hyg*., **2004**, *71*, 787-794.

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