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Mini Review

A novel mechanism for an old drug: Amphotericin B in the treatment of visceral leishmaniasis

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ABSTRACT

Visceral leishmaniasis (VL) is caused by various species of the genus *Leishmania*. Internalization of *Leishmania* into host cells is facilitated by a large number of receptors, and therefore no panacea is available for the treatment of leishmaniasis. We previously demonstrated the requirement of host membrane cholesterol in the entry of *Leishmania* into macrophages by cholesterol depletion using methyl-β-cyclodextrin (MβCD). We recently showed that leishmanial infection is inhibited upon sequestration of host membrane cholesterol using amphotericin B (AmB), considered as the best existing drug against VL. The reason for the antileishmanial activity of AmB is generally believed to be its ability to bind ergosterol in parasite membranes. Our recent results offer the opportunity to reexamine the mechanism behind the effectiveness of current AmB-based therapeutic strategies to treat leishmaniasis. We propose here a novel mechanism in which the effectiveness of AmB treatment could be partly based on its ability to sequester cholesterol in the host membrane, thereby abrogating macrophage–parasite interaction.

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1. Visceral leishmaniasis

Leishmania are protozoan parasites that are responsible for substantial public health problems, especially in tropical and subtropical regions. Leishmaniasis is a vector-borne disease, caused by various species of the genus *Leishmania*, which are obligate intramacrophage protozoan parasites. Leishmaniasis causes substantial public health problems, especially in tropics, subtropics and the Mediterranean basin, and is usually fatal if left untreated [1–3]. Leishmaniasis threatens about 350 million men, women and children in 88 countries around the world. Leishmaniasis is believed to be the third most prevalent vector-borne disease (the first two being malaria and lymphatic filiariasis) and it is estimated that 88 countries are leishmaniasis-endemic [3,4]. As many as 12 million people are believed to be currently infected, with about 1–2 million estimated new cases occurring every year [4]. Based on clinical syndromes, leishmaniasis is classified into four major types: cutaneous, muco-cutaneous, visceral (also known as kala-azar) and post-kala-azar dermal leishmaniasis. Among these, visceral leishmaniasis (VL) is fatal in the absence of treatment [3].

VL is caused by various leishmanial species in different geographical locations. It is caused by *Leishmania donovani* in the Indian subcontinent, Asia and Africa (in all age groups), and by *Leishmania infantum* or *Leishmania chagasi* in the Mediterranean region, southwest and central Asia, and South America (predominantly in children). Occasional cases of VL have been reported to be caused by *Leishmania tropica* in the middle east and *Leishmania amazonensis* in South America [5,6]. VL is usually associated with an incubation period of 2–6 months and is characterized by fever (accompanied by chills), weakness, night sweats, anorexia, weight loss, and enlarged lymph nodes, spleen and liver. Although these features are common in VL, certain variations in clinical symptoms are observed depending on the geographic location [3]. A particular feature, observed in case of VL patients in the Indian subcontinent, is hyperpigmentation that could have resulted in the name '*kala-azar*' (black fever in Hindi). During the advanced stage of the disease, abdominal distension and pain could be observed due to increased splenomegaly. There are 500,000 new cases of VL and more than 50,000 deaths from the disease every year [1,6]. However, these numbers could represent a lower limit since VL is often not diagnosed or reported, due to poor socioeconomic background of patients and their locations in remote rural areas [7]. The current increase in leishmaniasis throughout the world to epidemic proportion coupled with increasing incidence of the disease in developed countries, and emergence of VL as an important opportunistic infection among people with human immunodeficiency virus-1 (HIV-1) infection [8], have created an urgency to provide treatment for this disease.

2. Molecular events leading to leishmanial infection

Leishmaniasis is transmitted by the bite of the infected female sandfly (*Phlebotomous* spp.) when taking a bloodmeal from a host

Abbreviations: VL, visceral leishmaniasis; MβCD, methyl-β-cyclodextrin; AmB, amphotericin B; FITC, fluorescein isothiocyanate.

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[9,10]. The lifecycle of *Leishmania* has two distinct forms: an extracellular promastigote flagellar form found in the mid-gut of sandflies and an intracellular amastigote form that resides in phagolysosomes of mammalian (host) macrophages. Once in the bloodstream, promastigotes are internalized by dendritic cells and macrophages that subsequently transform into amastigotes by losing their flagella [3]. Entry of promastigotes into host macrophages involves multiple parasite–host interactions such as recognition of specific ligands on the parasite cell surface by receptors on the macrophage cell surface. A number of studies toward understanding the molecular mechanisms of parasite entry have led to the identification of several candidate receptors facilitating multiple routes of entry thereby highlighting the redundancy in the entry process [2,11,12]. These include membrane proteins present on the macrophage cell surface such as the mannose-fucose receptor, receptor for advanced glycosylation end products, the fibronectin receptor, the Fc receptor and complement receptors such as CR1 and CR3. The large number of different receptors responsible for the entry of the parasite into host macrophages makes it difficult to establish a unique therapeutic target for the treatment of leishmaniasis.

3. Membrane cholesterol: an important determinant in pathogen entry

The entry of *Leishmania* in particular and other intracellular parasites in general involves interaction with the plasma membrane of host cells. A number of previous studies have demonstrated the requirement of membrane cholesterol in host-pathogen interaction (reviewed in Refs. [13–22]). Cholesterol (see Fig. 1A) is an important component of higher eukaryotic cellular membranes and plays a crucial role in the function and organization of membrane proteins and receptors [23–25], some of which are necessary for parasite entry [26]. Our group was the first to demonstrate the requirement of host membrane cholesterol in the binding and internalization of *L. donovani* into macrophages using complementary approaches [27–29]. We previously showed that treatment of macrophages in culture with the cholesterol carrier methyl-β-cyclodextrin (MβCD) resulted in specific removal of membrane cholesterol, and a concomitant reduction in binding and subsequent infection by *Leishmania* promastigotes [27]. Interestingly, we showed that the binding/attachment of *E. coli* to macrophages did not change upon cholesterol depletion using MβCD. The latter observation shows that host–parasite interaction is specific. We also reported an accompanying reduction in the number of intracellular amastigote load of the parasite in cholesterol-depleted macrophages. Importantly, the reduction in binding of *L. donovani* promastigotes to cholesterol-depleted macrophages could be reversed by replenishment of cholesterol, thereby confirming the specific requirement of cholesterol in the infection process [27]. These results have been supported by the observation that membrane cholesterol is necessary for the entry of *L. chagasi* into host bone marrow macrophages through cholesterol-enriched caveolar domains [30].

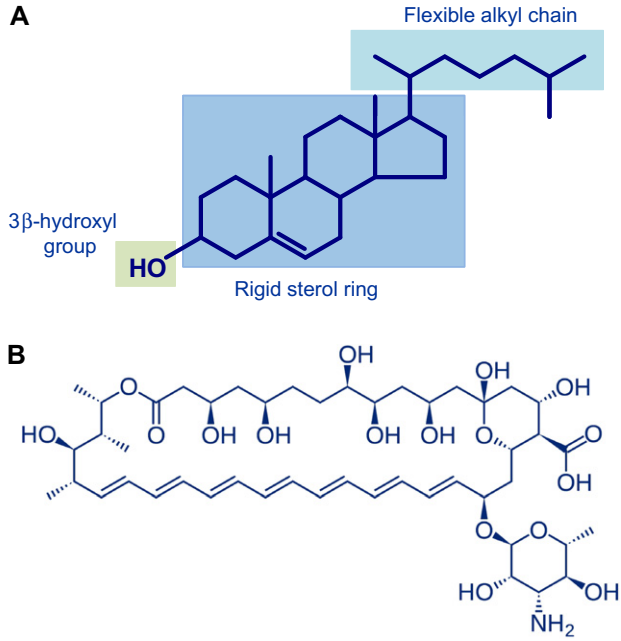

Fig. 1. (A) Chemical structure of cholesterol with the three structurally distinct regions (shown in different color boxes): the 3β-hydroxyl group, the rigid steroid ring and the flexible alkyl chain. The 3β-hydroxyl group is the only polar moiety in cholesterol, thereby providing it amphiphilic character. The hydroxyl group also helps to anchor cholesterol in the membrane. The rest of the molecule is hydrophobic and comprises of a planar tetracyclic fused steroid ring and a flexible isooctyl hydrocarbon tail. (B) Chemical structure of amphotericin B, a sterol-binding antifungal polyene antibiotic. Its molecular structure is characterized by a glycosylated lactone with an amphiphilic polyhydroxy region, a conjugated heptane chromophore and an amphoteric ion pair.

4. Amphotericin B: the drug of choice for the treatment of visceral leishmaniasis

Treatment of VL consists of specific anti-leishmanial drugs and aggressive management of any associated infections, anemia and malnutrition. For a long time, pentavalent antimonials have been the predominant drug for VL in many regions. These drugs are toxic and have adverse side effects and could lead to fatality [3,31,32]. The drug that replaced antimonials as the first line of treatment for VL is amphotericin B (AmB, see Fig. 1B). AmB and its formulations are increasingly being used and are considered as the best existing drugs against VL and have a 97% cure rate with no reported resistance [3,33,34]. Liposomal formulations of AmB [35], representing macrophage-targeted treatment, are often considered as most effective against VL [3,5]. AmB is a polyene antibiotic, first isolated in 1955 from *Streptomyces nodosus* from Venezuela [36]. It is a broad antimycotic agent and a highly antiparasitic one. It is the drug of choice for life-threatening systemic infections with fungi such as *Candida albicans* or *Aspergillus fumigatus*. Its extensive use in clinical practice is due to the morbidity and mortality brought about by fungal infections in immunodeficient (such as AIDS) patients. However, the usefulness of AmB is limited due to severe nephrotoxicity, which could result in kidney failure [37,38]. These side effects led to extensive research in formulations in the form of liposomes, emulsions and nanoparticles, all of which help reduce the amount of free AmB in blood stream, thereby reducing its toxicity [35,39–43].

It is important to understand the mechanism of action of any drug to further enhance its potency and/or to prevent major side effects. As mentioned earlier, AmB is considered to be the best available drug for the treatment of life threatening systemic fungal infections [44,45] and visceral leishmaniasis [46,47]. Extensive work has been carried out to understand the basis of action of AmB as an antimycotic and antiparasitic agent. It is generally believed that AmB is a membrane-active drug that forms channel-like structures (pores) spanning the lipid bilayer (see Fig. 2) [48–55]. In studies using liposomes or membranes isolated from target organisms and host cells, AmB is reported to form two types of ion channels with the formation of non-aqueous (cation-selective) channels preceding the formation of aqueous pores. Understanding the molecular steps involved in the formation of AmB channels and the role of membrane composition, bilayer thickness, presence of sterols (ergosterol or cholesterol) is important for designing better

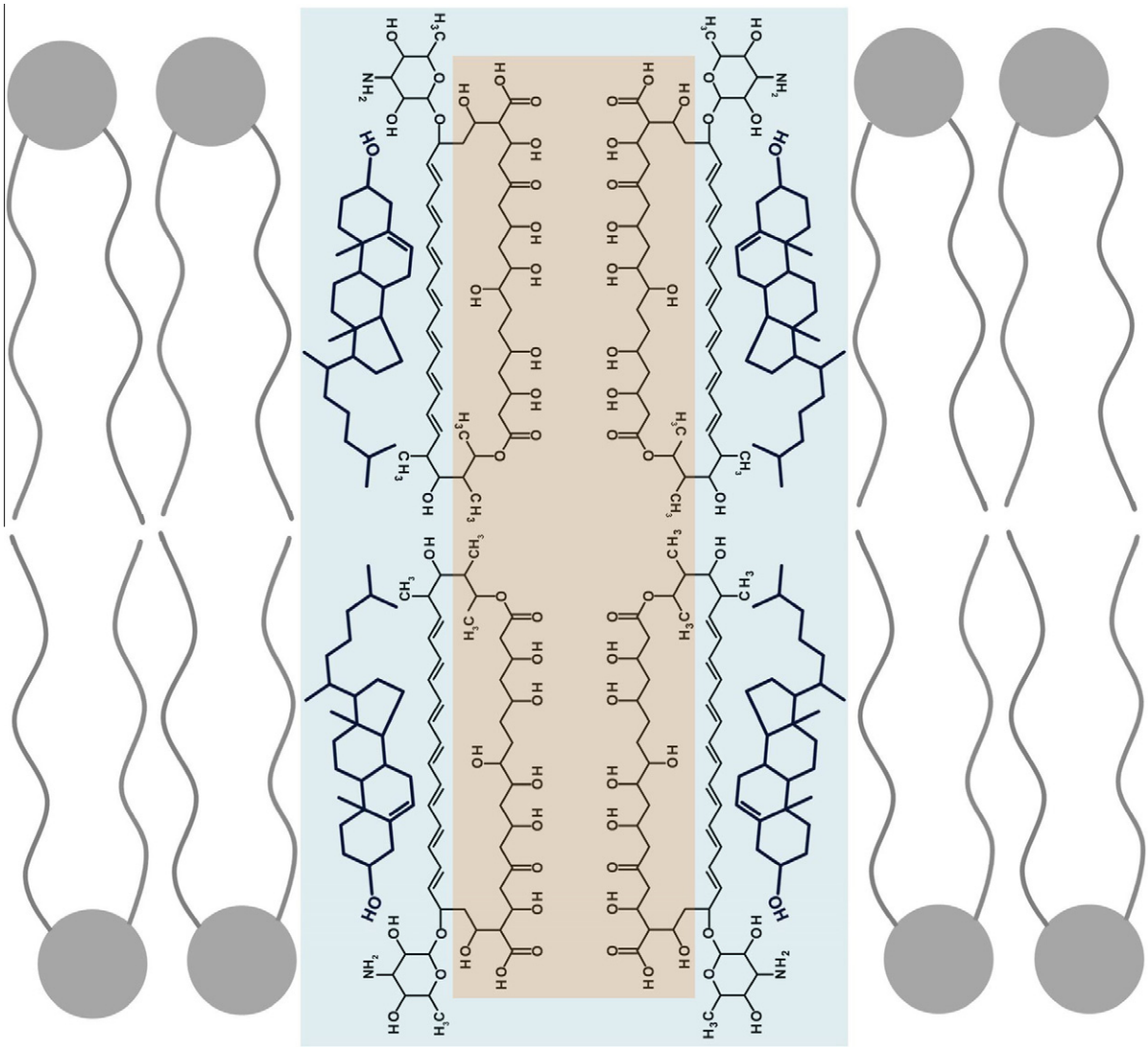

Fig. 2. A schematic representation of the possible organization of amphotericin B with membrane cholesterol. The membrane is shown as a bilayer of phospholipids and cholesterol, representative of typical eukaryotic membranes. The cross-section of the amphotericin B and cholesterol complex is shown in the membrane. Amphotericin B is an amphiphilic molecule with a hydrophobic side composed of polyene and a hydrophilic side composed of multiple hydroxyl groups that line the channel interior (shown in beige). The amphiphilic amphotericin B molecules spontaneously associate to form pores in the membrane. This pore structure is stabilized by cholesterol which interacts with the hydrophobic surface of amphotericin B. See text for other details.

strategies for the treatment with minimal side effects (recently reviewed in [56]). Membrane channels formed by AmB increase the permeability of cell membranes to ions and small solute molecules leading to cell death. It is well established that the presence of membrane sterols is essential for the complete manifestation of the channel-forming activity of AmB. The interaction of AmB with membrane sterols lead to the formation of transmembrane AmB channels which induce altered permeability to cations, water, glucose and affect membrane-bound enzymes [50,54,55]. The AmB–sterol complex is proposed to be a circular arrangement of ~8 AmB molecules interdigitated by equal number of cholesterol molecules. The outside of the complex is hydrophobic and the inside is hydrophilic due to the presence of the polar hydroxyl groups of AmB molecules (see Fig. 2). Two such complexes (half pores) would generate a pore which spans the membrane bilayer. The hydrophilic lumen of the channel is proposed to have a ~4 Å radius [54,55]. Although these general features of the interaction of AmB with membranes are known, studies on molecular mechanism of action of AmB have shown that the basis of biological action of AmB is complex. Detailed steps of the interaction of AmB with membranes have been addressed using molecular dynamics simulation [51–53].

As mentioned above, the usefulness of such a potent drug like AmB is limited due to severe nephrotoxicity induced by the drug [37,38]. The harmful side effects of AmB increase with increasing dosage, and therefore there is a limit on the amount of AmB that can be administered safely. Several strategies, including modification of the AmB molecule and changes in delivery systems, have been used to improve the therapeutic effectiveness of AmB and reduce its toxicity [57]. Among these, modification of the physical state of AmB is found to be the most promising one. The greater efficacy of liposomal AmB compared to Fungizone® (a mixture of AmB with a detergent, deoxycholate, in a phosphate buffer) formulation, was previously reported [57]. In order to further improve its potency and therapeutic value, numerous lipid formulations of AmB, with less toxicity than the parent compound, have been developed and studied (reviewed in [35,57,58]). The pharmacokinetics, toxicity and activity are clearly dependent on the type of AmB formulation. Some of these formulations such as liposomes, nanospheres and microspheres could result in higher concentra-

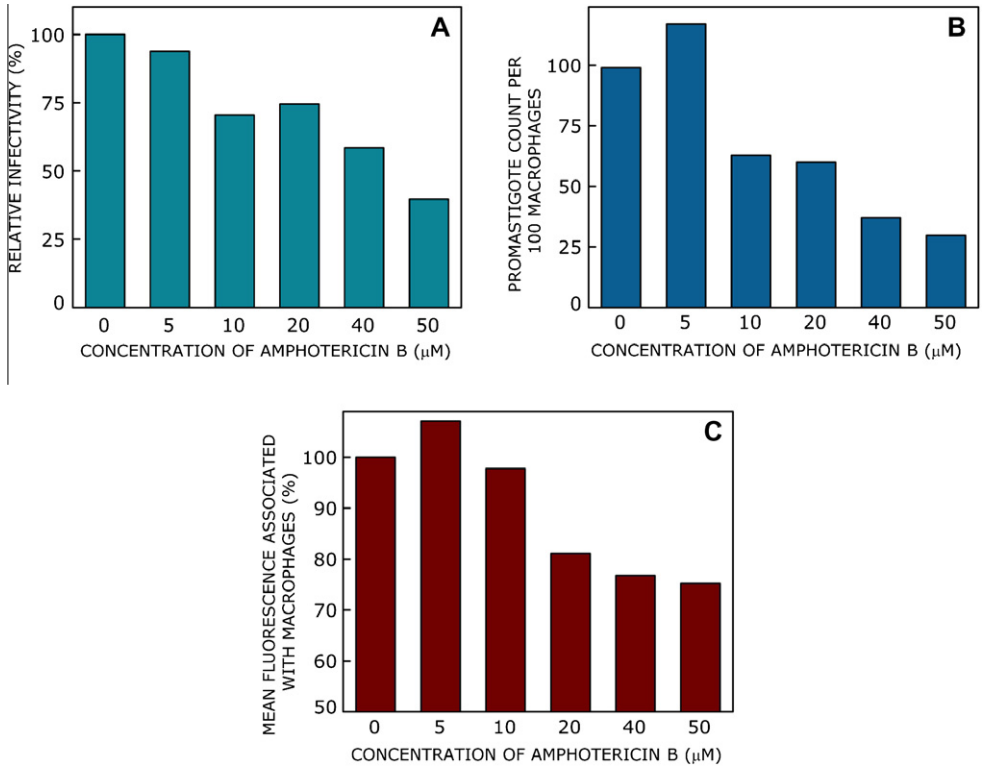

Fig. 3. Effect of amphotericin B on the extent of binding of *Leishmania* promastigotes to host primary macrophages. (A) Macrophages treated with increasing concentrations of AmB were exposed to radiolabeled *Leishmania* promastigotes and the extent of binding of the parasite to macrophages is shown. Values are normalized with respect to the mean counts per minute obtained for untreated (control) macrophages. (B) Promastigote count of infected macrophages treated with increasing concentrations of AmB. The corresponding count for untreated (control) macrophages is also shown. Macrophages pretreated with AmB show a considerable reduction in the number of promastigotes, as revealed by Giemsa staining. (C) The binding of FITC-labeled *Leishmania* promastigotes to J774A.1 macrophages treated with increasing concentrations of AmB is monitored by flow cytometry. Data for untreated (control) macrophages is also shown. Flow cytometric analysis shows a concentration-dependent reduction in the binding of promastigotes to host macrophages. Values are normalized to the fluorescence associated with untreated macrophages. Adapted and modified from Ref. [29]. See Ref. [29] for other details.

tions of AmB in the liver and spleen, but lower concentrations in kidney and lungs, thereby decreasing its toxicity. Several new AmB formulations with an improved efficacy/toxicity ratio have been marketed during the last few years [35]. Liposomal preparations of AmB are significantly superior to AmB emulsions or colloidal formulations in terms of bioavailability and side effects. Another advantage of such formulation is that AmB in plasma remains largely associated with liposomes for longer duration and is slowly released by the long-circulating liposomal delivery system [59].

5. Amphotericin B inhibits entry of *L. donovani* into host macrophages: reevaluation of the mechanism of leishmanicidal activity of AmB

As mentioned above, we [27] and others [30] have previously demonstrated the requirement of host membrane cholesterol in the binding and internalization of *Leishmania* promastigotes into macrophages. This was achieved by the use of MβCD which physically depletes cholesterol from membranes [60,61]. Treatment of macrophages in culture with MβCD resulted in the specific removal of membrane cholesterol and a concomitant reduction in binding and subsequent infection by *Leishmania* promastigotes [27]. If cholesterol is necessary for leishmanial infection, controlling membrane cholesterol availability by other means would affect infection. We recently tested this proposal by treating primary macrophages with AmB, a sterol-binding antifungal polyene antibiotic. AmB specifically interacts with membrane sterols (cholesterol in case of macrophages) to sequester it in the membrane, thereby effectively reducing the ability of cholesterol to interact with and exert its effects on other membrane components such as receptors (believed to be responsible for the entry of *Leishmania*). Interestingly, we observed that sequestration of cholesterol in the AmB-pretreated macrophage membranes (without physical depletion) is sufficient to inhibit leishmanial infection [29, see Fig. 3]. These results offer the interesting possibility of reevaluating the mechanism behind the effectiveness of current AmB based therapeutic strategies to treat leishmaniasis.

The antileishmanial action of AmB is believed to be due to its capability to bind ergosterol which is a major sterol in *Leishmania* [38,50]. Importantly, AmB also binds cholesterol with comparable affinity [62,63]. In case of *in vivo* AmB treatment, both host and parasite membranes are exposed to AmB. *We propose the novel mechanism that the effect of AmB treatment could be due to a combination of its interaction with both sterols i.e., ergosterol of Leishmania and cholesterol of host macrophages.* On a broader perspective, these results offer the possibility of reexamining the mechanism behind the effectiveness of current therapeutic strategies that employ sterol-complexing agents such as AmB to treat leishmaniasis. Although the development of AmB as a therapy against leishmaniasis has its origin in the discovery that it is a potent leishmanicidal agent [64,65], it is possible that its effectiveness *in vivo* is partly based on its ability to sequester cholesterol in the host membrane, thereby abrogating macrophage–parasite interaction. Future research could further explore this issue and may lead to novel therapeutic strategies against leishmaniasis based on fine-tuning of cholesterol complexing property of AmB.

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