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Liposomal formulations of amphotericin B: differences according to the scientific evidence

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ABSTRACT

This article presents an overview of the characteristics of liposomes as drug carriers, particularly in relation to liposomal formulations of amphotericin B. General features regarding structure, liposome-cell interactions, stability, encapsulation of active substances and elimination of liposomes are described. Up to the present time extensive efforts to produce similar or bioequivalent products of amphotericin B formulations, in particular in the case of liposomal amphotericin B, have been unsuccessful in spite of having a very similar composition and even an apparently identical manufacturing process. Guidelines for the development of generic liposomal formulations developed by the FDA and EMA are also summarized. Based on the available evidence of the composition of liposomes, any differences in the manufacturing process even if the same lipid composition is used may result in different final products. Therefore, it seems unreasonable to infer that all amphotericin B liposomal formulations are equal in efficacy and safety.

Key words: Amphotericin B, Liposomal formulations, Liposomes, Drug carriers.

Formulaciones liposómicas de anfotericina B: diferencias basadas en la evidencia científica

RESUMEN

Este artículo presenta una visión general de las características de los liposomas como vehículos portadores de fármacos, especialmente en relación con las formulaciones liposómicas de anfotericina B. Se describen los aspectos generales relativos a la estructura de los liposomas, interacciones del liposoma con la célula, estabilidad, encapsulación de los principios activos y eliminación de los liposomas. Hasta el momento presente todos los esfuerzos para producir productos similares o bioequivalentes de formulaciones de anfotericina B, especialmente en el caso de la anfotericina B liposómica has resultado infructuosas, a pesar de tener una composición similar e incluso un proceso de producción idéntico. Asimismo, se resumen las guías elaboradas por la FDA y EMA para el desarrollo de formulaciones liposómicas genéricas. De acuerdo con la evidencia disponible sobre la composición de los liposomas, cualquier diferencia en el proceso de producción, incluso usando la misma composición lipídica puede determinar diferencias en los productos finales. Por tanto, no parece razonable inferir que todas las formulaciones liposómicas de anfotericina B son iguales en eficacia y seguridad.

Palabras clave: Anfotericina B, formulaciones liposómicas, liposomas, portadores de fármacos.

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INTRODUCTION

Early recognition of the high frequency of adverse effects of intravenous administration of deoxycholate formulation of amphotericin B (AmB-dox) together with the severity of illness of patients in which this therapy was usually prescribed, prompted the development of multiple actions directed to reduce drug toxicity, while maintaining the indispensable efficacy.

It was initially described that dilution of the antifungal agent in commercial lipid formulations used in parenteral nutrition regimens may allow to achieve this goal[1-3]. The potential usefulness of prolonged intravenous administration even in continuous infusion was also reported[4-7].

At the same time, new lipid-associated formulations were developed, among which the liposomal preparation of amphotericin B (AmBisome®) was particularly relevant for its lower renal toxicity.

Currently, there are initiatives aimed at forming homogeneous pharmacological classes with drugs considered similar and intended to be interchangeable, or even the development of generic drugs. These apparently simple and justified practices in almost any pharmacological group, present *a priori* very special connotations in the case of liposomal formulations of amphotericin B due to the particular characteristics of such

formulations, the description of which is the main purpose of the present review.

LIPOSOMES

Liposomes are composed of one or several concentric lipid layers that encapsulate an aqueous compartment. The concentric arrangement which is facilitated by the presence of phospholipids produces small spheres-like formations. The size of these nearly spherical lipid vesicles can range between 50 and 450 nm.

Phospholipids consist of a molecule of glycerol, two fatty acids (1,2-diacylglycerol) and a phosphate group (figure 1). The phosphate group along with the glycerol group makes the head of the phospholipid hydrophilic, whereas the fatty acid tail is hydrophobic. Thus phospholipids are amphipathic: a molecule with a polar end and a hydrophobic end. When phospholipids are in an aqueous solution they will self-assemble into micelles or bilayers, that is, structures that exclude water molecules from the hydrophobic tails while keeping the hydrophilic head in contact with the aqueous solution[8].

Depending upon the structure, there are two types of liposomes: unilamellar and multilamellar liposomes. Unilamellar vesicles (ULV) have a single phospholipid bilayer sphere (average diameter between 50 and 250 nm), enclosing aqueous solution (figure 2), which make them suitable to encapsulate water-soluble drugs. Multilamellar vesicles (MLV) consist of two or more concentric bilayers with higher particle size and entrap lipid soluble drugs.

Hydrophobic interactions between lipid layers and hydrogen sources, and polar interactions between water molecules of the aqueous solution and polar heads are responsible for strengthening of the structure and, therefore, of the liposome stability. The final organization of lipids within the liposome depends on the nature, concentration, temperature and the adopted geometric form.

ULV liposomes exhibit a much faster release rate of entrapped molecules than MLV liposomes because it is more difficult for any molecule to pass through several lipid layers than through a single one[9-10].

Active therapeutic principles encapsulated into liposomal carriers should be released to exert their pharmacological effects usually after destruction of the liposome, so that stability of liposomes is crucial to ensure a stable drug product.

Cholesterol is an important factor that may affect liposomal stability, reducing permeability, increasing *in vivo* and *in vitro* stability and inhibiting transference between high-density (HDL) and low-density (LDL) lipoprotein fractions. These actions justify the use of cholesterol in different liposomal formulations, including amphotericin B[11].

Stability of liposomes also depends on the nature of the phospholipid molecules contained in their structure. Pure phospholipid vehicles can pass from the gel phase to the liquid-crystalline phase as the room temperature is increased. This transition occurs in a wide range of transition temperatures, which may range between 60°C for dipalmitoyl phosphatidyl etanolamina and -20°C for dioleoylphosphatidylcholine bilayer. In this respect, it should be noted that some of the lipid formulations currently available contain the phospholipid, dimyristoyl phosphatidylcholine, which has a fusion temperature of 23°C, therefore, with the possibility to release components quickly, among which amphotericin B, at body temperature. By contrast, the use of some phospholipids with high transition temperature, such as phosphatidylcholine facilitates liposomal stability[12].

The use of hydrophilic carbohydrates, monosialogangliosides type polymers, or even polyethylene glycol (PEG) in

Figure 1 General structure. Phospholipid.

liposomes, also provides stability and in case of the polyethylene glycol, this compound reduces uptake by the reticuloendothelial system (RES), thus prolonging the blood circulation time[13,14].

There are different methods of liposome preparation, the description of which is beyond the objective of this article, but the type of method used provides a final specific result, which is conditioned by different factors, such as physicochemical characteristics of the components and the active principle, concentration, dispersion liquid medium, particle size and half-life, as well as foreseen large-scale manufacturing costs. The process of preparation depends on these variables and even when the same components are used, the fact that the characteristics of the final product will be identical cannot be guaranteed[15-17].

LIPOSOMES AND PHARMACOKINETICS

Encapsulation of active substances into the lipid bilayer protects them against naturally occurring phenomena, such as chemical inactivation, enzymatic degradation and immunological neutralization. Therefore, liposomes prevent a drug from being metabolized prior to reaching target tissues, and simultaneous they minimize exposure of healthy tissues to the encapsulated drug during its circulation in the blood. All of these effects contribute to provide drugs used in liposomal formulations of pharmacological characteristics that are different from that of conventional formulations, which finally result in a notable increase of the therapeutic index of the active principle, which to a large extend will depend upon the delivery rate.

As previously stated, the process of release from the liposome depends on multiple factors, some of them still poorly understood, in particular the nature of the lipid bilayer, the size of the drug molecules, their lipid solubility and the capacity of interaction with lipid membranes.

The encapsulation efficiency of a molecule in a liposome depends on its polarity and partition coefficient, which also determines its localization in the liposomal membrane. If a drug is hydrophobic in nature, it resides in the acyl hydrocarbon chain of the liposome, and hence delivery properties are dependent on the characteristics of the acyl chains of the liposome and, at the same time, of the liposolubility of the active principle. On the other hand, if a drug is polar/hydrophilic, it tends to localize in the aqueous core or adjacent to the water–lipid interface, near the polar head groups of the liposome[18].

Liposomes as any other biological membrane, have a high permeability to lipid-soluble drugs and a low permeability to water-soluble drugs. Highly hydrophilic drugs are retained in the aqueous compartment of liposomes and slowly released over several hours or days. On the contrary, hydrophobic molecules may be linked to a fatty acid chain, which is inserted into the phospholipid bilayer and therefore are more easily released[19-20].

Elimination of liposomes takes place in different ways. One way involves absorption of plasma proteins on the surface of liposomes and then their recognition by the reticuloendothelial system (RES). This event results in the excretion of liposomes at the hepatic level and its subsequent metabolism by Kupffer cells. In the second way, liposomes are metabolized by splenic macrophages. Finally, after their accumulation, they are metabolized and eliminated by the target tissues. However, it is unclear whether capture of liposomes by the RES represents a true advantage in the treatment of diseases, except in the case of specific infections, in which the high concentration of antimicrobial agents in the RES can help to treat infective pathogens[21,22].

Liposomes can be adsorbed into the membrane of cells, where the lipid bilayer of the carrier is degraded by enzymes, such as lipases. This leads to the release of the active ingredients into the extracellular fluid, where they can diffuse through the cell membrane and cytoplasm.

Another mechanism requires the fusion of the liposomal membrane with the plasma membrane of the target cell, and this phenomenon causes the release of liposomal content directly into the cytoplasm.

The third and probably the most frequent mechanism of

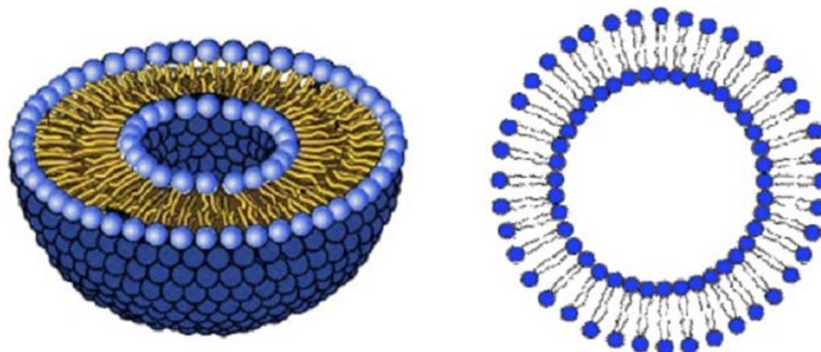

Figure 2 | **Unilamellar liposome.**

liposome interaction with a cell is receptor-mediated endocytosis. This process is only possible for vesicles of less than 150 nm in diameter. Phagocytosis can also occur, but involves liposomes of a diameter larger than 150 nm.

Liposomal size is also an important characteristic. It has been shown that liposomes smaller than 100 nm in diameter interacted less with plasma proteins, evaded capture by the RES and had longer elimination half-life in the blood[23].

Larger liposomes are eliminated more rapidly from blood circulation as being captured, deposited and probably destroyed, at least in part, by the RES.

At the present time, the ideal size of a liposome for appropriate drug delivery at the target site should be 50-100 nm in diameter[24,25].

Small cholesterol containing liposomes, such as AmBisome®, and/or PEGylated showed a remarkable improved stability and circulation times. During their circulation in the blood, liposomes are bind to plasma proteins, mainly opsonins and HDLs and LDLs. Opsonins include various protein types, like immunoglobulins and fibronectin, which help RES recognize and eliminate liposomes. Blood carrying HDL and LDL interacts with liposomes and reduces their stability. The interaction with lipoproteins causes lipid transfers and rearrangements on the surface of liposomes. This frequently induces lipid depletion, liposome breakdown and rapid release of the substance to the plasma[26].

Also, liposome–cell interaction is strongly influenced by the nature and density of the charge of the liposomes surface. The liposomes can include charged components that confer them an overall neutral, positive, or negative charge[27-31]. Lack of surface charge (neutral liposomes) increases the aggregation of liposomes and reduces their physical stability. Moreover, neutral liposomes do not interact significantly with cells, and this causes drug release from the liposomes in the extracellular space[32].

Negatively charged liposomes are generally constituted by anionic lipids, such as dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol. Negative liposomes are less stable than neutral and positive liposomes when injected into the blood circulation. In fact, anionic liposomes rapidly interact with the biological system subsequently to their opsonization with complement and other circulating proteins. Such an interaction has at least two acute consequences: a rapid uptake by the RES, and toxic effects, such as pseudoallergy. Negative or anionic liposomes are exceptionally used as drug-delivery systems for intravenous administration, and seems more suitable to take advantage for transdermal drug delivery, due to their enhanced penetration properties through the skin[28,30,33,34].

Cationic liposomes (CLP) (positively charged liposomes) contain different types of specific phospholipids and it seems that the endocytic pathway is the preferential route of internalization[35,36].

When a therapeutic agent is loaded into liposomes, it adopts the carrier's pharmacokinetics until it is delivered. As a result, liposomes modify both the tissue distribution and the rate of clearance of the loaded drug.

NEW LIPOSOMAL FORMULATIONS

Up to the present time extensive efforts to produce similar or bioequivalent products of amphotericin B formulations, in particular in the case of AmB-Lip, have been unsuccessful in spite of having a very similar composition and even an apparently identical manufacturing process.

A study that compared the efficacy and toxicity of two liposomal formulations of amphotericin B, AmBisome® and Lambin®, in a model of *Aspergillus fumigatus* infected mice provided evidence of the differences between two formulations of the same active substance and identical lipid composition but with different manufacturing processes[37]. The results showed that Lambin® was more toxic than AmBisome® based on the red blood cells (RBC) potassium release assay. In addition, intravenous dosing in uninfected mice given a single 50 mg/kg dose was associated with 80% mortality for Lambin® and 0% for AmBisome®. With a 10 mg/kg dose, survival in *Aspergillus fumigatus* infected animals was 30% for Lambin® and 60% for AmBisome®. The administration of AmBisome® at 10 or 15 mg/kg, or 15 mg/kg of Lambin® to infected animals lowered fungal burden in bronchoalveolar lavage (BAL) and lung tissue samples. The authors of this study indicate that the process used for loading amphotericin B is crucial regarding the composition of the carrier and has a relevant effect on the pharmacokinetic and pharmacodynamics properties of the final product[37].

In another study, the physical, antifungal, pharmacokinetic and toxic properties of two liposomal amphotericin B products, AmBisome® and Anfogen that have the same chemical composition but are manufactured differently, were compared[38]. *In vitro* tests included determinations of the minimal inhibitory concentrations (MICs) and the concentrations causing the release of 50% of the intracellular potassium from red blood cells (K_{50} values) to assess toxicity. *In vitro* K_{50} values were significantly lower for Anfogen (0.9 µg/ml) than for AmBisome® (20 µg/ml). Also, the LD_{50} of AmBisome® was >100 mg/k *vs.* 10 mg of Anfogen/kg. The median particle size was 77.8 nm for AmBisome® and 111.5 nm for Anfogen. The incidence of renal tubular necrosis in uninfected and infected mice was significantly higher in those given Anfogen as compared to those treated with AmBisome®. At the same time, AmBisome® at 7.5 or 15 mg/kg was also more efficacious than 7.5 mg of Anfogen/kg for the treatment of pulmonary aspergillosis, both in clinical-related variables and reduction of colony-forming units (CFU) per gram of lung[38]. These results showed that AmBisome® and Anfogen were not comparable (with AmBisome® being 10-fold less toxic than Anfogen), despite the fact that the chemical composition of the products was the same. The association between the carrier and the active agent, amphotericin B, can be significantly altered by the processes used to prepare the product, and this association is critical for obtaining the desired therapeutic index of the carrier-drug preparation[38].

Table 1	Quality parameters of liposomal lipid formulations. Physicochemical properties
	- Description, source and characteristics of the manufacturing process, impurity profile, isomers and stability characteristics of the lipid components. - Quality, purity and stability characteristics of other critical excipients. - Identification and control of key intermediates in the manufacturing process. - Active substance/lipidic component ratio with the acceptability range. - Liposome morphology, mean size and size distribution, and aggregates. - Fraction of encapsulated active substance (amount of free/entrapped). - Stability of the active substance, lipids and functional excipients in the finished product, including quantification of critical degradation products. - Drug substance release rate from the liposome in physiologically/clinically relevant media. - Stability on storage. - Stability under proposed in-use conditions. - Process for reconstitution and/or pharmacy preparation.
	Depending on the specific function of the liposomal formulation other parameters should be evaluated: - Maintenance of liposomal formulation integrity in plasma. - Characterization of lipid bilayer phase transition behavior. - Determination of liposomal charge. - pH of internal compartment for pH-gradient loaded liposomes. - Characterization of physical state of the active substance inside the liposome. - Distribution of drug substance within liposome. - Specific characterization for conjugated liposomal formulations (quality, purity, type of linkage chemistry, molecular weight, size, stability of conjugation, etc.).

Guidelines for the development of generic liposomal formulations

Because of the special characteristics of liposomal formulations already described in this review, the federal agency Food and Drug Administration (FDA) of the United States and the European Union regulatory agency for the evaluation of medicinal products (EMA) have reported specific recommendations for the development of generic drug products using liposomal formulations[39,40].

EMA has provided guidelines on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product. Central aspects of the document include pharmaceutical quality and clinical and non-clinical pharmacology requirements, some of which are here summarized.

Pharmaceutical quality. It is established that the quality of liposomal formulations is critical because it may have a major impact on the *in vivo* pharmacokinetic (PK) and pharmacodynamics (PD) properties in relation to some of the following mechanisms:

- Released rates of the active substance from liposomes can affect PK and PD and therefore the efficacy and tolerability of the medicinal product.
- The entrapped active substance is not biologically active and is protected from degradation whilst it is entrapped in the liposome.
- The PK of the encapsulated active substance is controlled by the PK of the carrier (liposomal formulation), which is influenced by the physicochemical properties of the liposomes.
- The formulation may affect uptake and tissue distribution.

In this regard, it is essential to establish the pharmaceutical comparability of formulations based on the main condition that the quantitative and qualitative composition has to be identical to the reference product. The parameters that should be evaluated for quality characterization of liposomal formulations are described in table 1.

Non-clinical studies. In general, non-clinical studies should be performed before clinical studies and should include comparative investigation of pharmacokinetics (including tissue distribution) of single and multiple doses, toxicology and pharmacodynamics.

Non-clinical pharmacokinetic studies are aimed to demonstrate the similarity of distribution and elimination of both free and encapsulated products at single and multiple doses. Non-clinical pharmacodynamics studies should include demonstration of the similarity in pharmacodynamic response using appropriate *in vivo* models, as well as *in vitro* tests capable of characterizing any interaction between liposomes and target cells or other cells where the interaction is toxicologically relevant.

Clinical studies. Clinical studies should include comparative pharmacokinetic studies and assessment of efficacy and tolerability. Pharmacokinetic studies should assess pharmacokinetic characteristics of the encapsulated and unencapsulated

drug substance to allow assessment of the rate at which active substance is released from the liposomes. For the acceptance criteria of similarity, the 90% confidence intervals of C_{max} and AUCt rations should be within 80-125%. The necessity to perform studies to demonstrate efficacy and/or tolerability is usually decided on a case-by-case basis depending on the results of the non-clinical models and clinical pharmacokinetic data.

CONCLUSIONS

Clearly the history of AmBisome® is still unfinished today, and after having being used for more than 20 years, the specific circumstances that concur in this drug and how differences in behavior compared to other amphotericin liposomal formulations remains unknown. The characteristic behavior of this amphotericin formulation appears to be related to composition of the liposome, and for this reason, any difference in the manufacturing process even if the same lipid composition is used ends up in generating notable differences in efficacy and tolerability, as can be deduced from the accumulated evidence with some formulations that intended to be generic and did not succeed. These results prompted the development of EMA and FDA recommendations for the assessment of different aspects related to pharmaceutical quality, non-clinical and clinical studies in the process of development of any generic liposomal formulation of amphotericin. Based on the landscape evidence here presented, it seems unreasonable to make inferences that all amphotericin B liposomal formulations are equal in efficacy and safety.

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