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Development and characterization of oral liposomes of vegetal ceramide based amphotericin B having enhanced dry solubility and solubility

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ABSTRACT

Despite the development of new antifungal, amphotericin B remains one of the most effective agents in the treatment of systemic fungal infections. Many patients exhibit nevertheless intolerance to amphotericin B at higher dosages and parenteral formulations present unlike per os ones, associated risks and high care cost. Free amphotericin B *per os* showed however an apparently poor absorption. In this study, we evaluate the potential of amphotericin B liposomes formulated with vegetal ceramides for oral administration. Ceramides, one of the constituents of cellular cytoplasmic membranes, constitute an important element in the construction and stability of their lipid bilayer. To fulfill this objective, vegetal ceramides, composed essentially of glucosylceramides, were firstly incorporated in various liposome preparations, entrapping or not amphotericin B, in comparison with phosphatidylcholine liposomes. Then, these preparations were introduced in an "Artificial-Stomach–Duodenum" model to improve their stability for oral administration. The formulation of amphotericin B liposomes containing ceramides presented a mean hydrodynamic size of about 200 nm. We showed also that cholesterol and phospholipids are required to prevent drug leakage and to obtain lamellar structure respectively. In "Artificial-Stomach–Duodenum" model, ceramides conferred to liposomes better membrane stability. In addition, ceramides did not alter their drug encapsulation yield being by 75%. This could be explained by the fact that ceramides as we proved, limited the detergent effect of bile salts on liposome membranes.

1. Introduction

In developed nations, opportunist disseminated fungal infections are on the rise, affecting immunocompromised patients with e.g. cancer, organ transplant recipients or HIV/AIDS. In such patients, invasive fungal infections may account for as many as 30% deaths. Despite the development of new antifungal, amphotericin B remains one of the most effective agents in the treatment of systemic fungal infections. However, many patients exhibit intolerance to amphotericin B, particularly at higher dosages [1]. The most serious adverse reaction reported with amphotericin B therapy is nephrotoxicity with sodium depletion [2,3]. A variety of parenteral formulations consisting of encapsulate drug in different vesicles have been studied to prevent these side effects [4–7]. Entrapping amphotericin B in liposomes or various lipid formulations seems to be the best strategy to reduce amphotericin B nephrotoxicity and improve drug delivery to target sites [8,9]. While effective, the limitations of amphotericin B parenteral administrations are the safety issues associated with this administration (e.g. infections of the indwelling catheter or patient chills and shaking due to erythrolysis) and high drug cost.

An oral formulation of amphotericin B is desirable to treat infections and provide or prophylactic therapy for high-risk patients. This route will also increase compliance and prevent parenteral side effects. Free amphotericin B per os showed however an apparently poor absorption [10]. Recently several oral formulations were described such as amorphous drug nanoparticles [11], carbon nanotubes [12], chitosan microparticles [13], cubosomal nanoparticles [14], nano-emulsions [15], gelatin nanoparticles [16], polymeric nanoparticles [17] or solid lipid nanoparticles [18].

Liposomes could also be an interesting solution. Indeed, this vesicle is reputed to have high loading capacity for hydrophobic drug, and by appropriate choice of particle size and surface characteristics, some degree of selectivity is possible [19]. Therefore, incorporation of amphotericin B into liposomes will alter its pharmacokinetic profile leading to changes in tissue distribution, antifungal activity and importantly, tolerability. Unilamellar and multilamellar vesicular structures can be formed from a variety of materials such as amphipathic lipid substances including *e.g.* saturated and unsaturated fatty acids or synthetic ionic and nonionic surfactants [20]. The vesicles, fabricated from these materials, particularly when combined with cholesterol, can entrap hydrophilic and lipophilic drugs. Lopez-Berestein et al. were the first to

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report the use of multilamellar liposomes to deliver amphotericin B [21]. A second liposomal product, Ambisome®, incorporates amphotericin B into small unilamellar vesicles [8,22,23]. Amphotericin B can also be efficient into lipids without vesicular structures. The various lipid formulations tested included amphotericin B lipid-based formulations, amphotericin B lipid complexes and amphotericin B cholesterol sulfate colloidal dispersion [8,24,25].

Ceramides, a combination of sphingosine and amide-linked fatty acid, are one of the constituents of cellular cytoplasmic membranes. They constitute an important element in the construction of the lipid bilayer, and are thought to maintain the multi-bilayer organization of lipidic barrier. In point of fact of their structural characteristics, vegetal ceramides are "rigid" molecules, therefore least exposed to the bile salts and digestive enzyme activities comparative to phospholipids.

The aim of this study was to investigate if to entrap amphotericin B in vesicles, another type of lipids as vegetal ceramides could be used for oral administration and could confer a stability of membranes in digestive medium. To fulfill this objective, vegetal ceramides, composed essentially of glucosylceramides, were firstly incorporated in various liposome preparations, entrapping or not amphotericin B, in comparison with phosphatidylcholine liposomes. Then, these preparations were introduced in an "Artificial-Stomach–Duodenum" model to improve their stability for oral administration.

2. Materials and methods

2.1. Materials

Amphotericin B was obtained from Squibb (Paris, France). Egg yolk phosphatidylcholine (EPC) was obtained from Lucas Meyer Cosmetics (Versailles, France). L-α-Distearoyl phosphatidylcholine (DSPC) and Cholesterol (CHO) were obtained from Sigma Aldrich (Saint-Louis, MO, USA). Ceramides (CER) containing glycosylceramides (80%), gliadine (10%) and apolar lipids (10%) were extracted and purified from wheat by Inocosm (Châtenay-Malabry, France). All other chemicals were commercially available products of analytical grade.

2.2. Preparation of liposomes

Preparations of various formulations: EPC:CHO (molar ratio 2:1); EPC:CHO:CER (4:2:1); CER:CHO (2:1), CER:CHO:EPC (4:2:1), CER:CHO:DSPC (4:2:1) and amphotericin B entrapped into liposomes of formulation EPC:CHO (2:1) and CER:CHO:EPC (4:2:1) were prepared by a modification of a patented injection method [26].

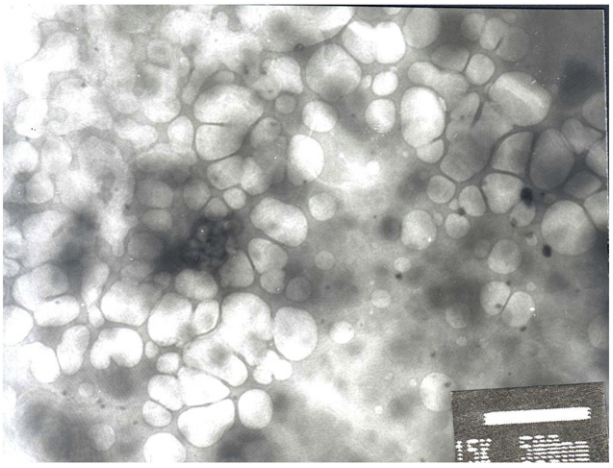

Fig. 1. Transmission electron microscopic observation from negatively stained preparations of ceramide mixture CER:CHO (2:1 ratio molar). Vesicles have an external membrane and a mean diameter of 100–300 nm.

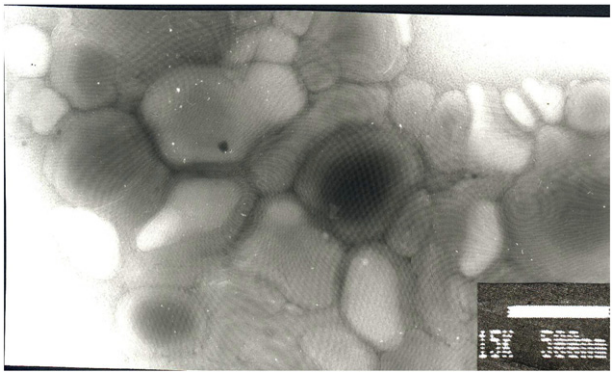

Fig. 2. Transmission electron microscopic observation from negatively stained preparations of liposomes CER:CHO:EPC (4:2:1, ratio molar). Vesicles were multilamellar liposomes of about 200–500 nm.

Briefly, individual lipids (3.5 mg/ml) and/or ceramides (3.5 mg/ml) were dissolved in a methanolic solution with or without 0.5 mg/ml amphotericin B to obtain respectively either amphotericin B entrapped into liposomes or blank liposomes. This organic phase, placed in a syringe, was then added to the aqueous phase (0.9% NaCl solution) at 25 °C with a constant flow of 1 ml/min. At the end of this step, organic solvent was removed by using an ultra-turrax at 24 000 rpm during about 10 min (IKA, Staufen, Germany).

2.3. Amphotericin B content

The amount of amphotericin B entrapped into liposomes was determined by the reverse-phase High Performance Liquid Chromatographic Method (HPLC). A column (Spherisorb ODS 2 SHANDON, 5 μm), an auto-injector (model JASCO AS 950) and a multichannel photo UV detector (model MERCK L-3000) operated at 408 nm were used for drug quantification. The mobile phase consisted of 34% of acetonitrile, 10% of tetrahydrofuran (THF), 0.1% of triethylamine and 55.9% of distilled water at pH = 5.2 and at a flow rate of 1 ml/min. Under these conditions, the retention time of amphotericin B was 6.8 min.

The liposome suspensions were diluted in methanol (1/1000). Aliquots (400 μl) of this diluted suspension were centrifuged for 10 min at 600 ×g and the supernatants were separated, diluted in methanol (1/1000) and analyzed (initial amount amphotericin B dosed) by HPLC. Aliquots (400 μl) of liposome suspensions were centrifuged for 60 min at 100,000 ×g, and the supernatants were separated, diluted in methanol (1/1000) and analyzed by HPLC (free amount amphotericin B dosed). The amount of amphotericin B associated with liposomes (% inclusion) was determined from the ratio:

$$\%\,\text{inclusion} = \frac{(\text{initial amount dosed} - \text{free amount dosed}) \times 100}{\text{initial amount dosed}}.$$

2.4. Size distribution evaluation

Mean hydrodynamic diameters were obtained by dynamic light scattering using a DLS instrument (N4MD, Beckman Coulter, Roissy

Table 1
Comparison of liposome mean size and amphotericin B (amB) inclusion yield of different formulations. Each measurement was in triplicate.

Composition (molar ratio)	Mean size (nm)	% of inclusion of AmB
EPC:CHO (2:1)	354 ± 7	75.4 ± 1.97
CER:CHO:DSPC (4:2:1)	200 ± 10	75.0 ± 2.00
CER:CHO:EPC (4:2:1)	171 ± 8	75.0 ± 3.08
CER:EPC (4:1)	171 ± 8	44.0 ± 3.61