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European Journal of Pharmaceutics and Biopharmaceutics 69 (2008) 908–922
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Pre-formulation of liposomes against *Helicobacter pylori*: Characterization and interaction with the bacteria

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Received 27 July 2007; accepted in revised form 7 January 2008
Available online 31 January 2008

Abstract

This paper deals with the formulation of targeted liposome against *Helicobacter pylori*. We describe the characterization of liposomes loaded with antimicrobial agents (ampicillin and metronidazole) and the quantification of the interactions between such formulations and bacteria. If the encapsulation rate of ampicillin seems not strongly affected by the change of phospholipidic composition, the encapsulation of metronidazole drastically decreased in epikuron 170 liposomes compared to DPPC ones. Furthermore, as observed with X-ray diffraction measurements, the presence of metronidazole results in the disorganisation of the phospholipid bilayers. Concerning the liposome–bacteria interactions, it has been observed that the incorporation of fucosyled glycolipids in the vesicle membrane leads to liposomes that are able to interact with the bacteria either in their spiral or in their coccoid forms. Since coccoid forms are occasionally found in vivo, their recognition by the liposomes we have formulated seems promising in the fight against *Helicobacter pylori*.

Keywords: Liposome; *Helicobacter pylori*; Glycolipid; Targeting; BabA2

1. Introduction

Helicobacter pylori was discovered by Warren and Marshall in 1982 (2005 Nobel prize), and confirmed as a pathogen at the end of the 80s. In 1994, the WHO classified the microorganism as type I carcinogen because of the gastric cancers and MALT (mucosa-associated lymphoid tissue) lymphomas which can occur after a chronic infection. The worldwide prevalence of this bacterium is high [1–3] and the present eradication rate does not reach the objective defined by WHO, i.e. 90% [4]. Today, the standard treatment to cure *H. pylori* infection is a 7-days tri-therapy based on two antibiotics (amoxycillin and clarithromycin) and one proton pump inhibitor (omeprazole, lansoprazole, or pantoprazole) or occasionally, an association of bismuth salt with one or two antibiotics. However, because of the high level of antibiotic resistance to *H. pylori* and the poor patient compliance [5], new drugs with better effectiveness and simpler regimen are required. Indeed, *H. pylori* is sensitive to many antibiotics but a number of them cannot be used in acidic medium [6]. However, due to the urease activity, the close environment of the bacterium is neutralized by the production of ammonia and carbon dioxide [7,8]. A release of the active substance in the nearby of

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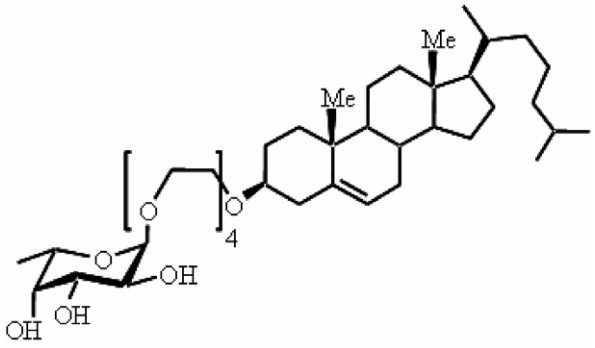
doi:10.1016/j.ejpb.2008.01.018

the bacterium could overcome the problem of acidity. Encapsulation of an active substance could be a good approach, by offering a protection against the stomachal acidity. In a seek for a non toxic vector able to encapsulate a wide range of drugs and easily modifiable at the surface, we have identified liposomes as good candidates for this purpose. Indeed, incorporation of specific ligands at liposome surface, could allow a targeting to *H. pylori* and would allow an increased stomachal retention time of the drug. The other advantage of liposomes is their similarity with cell membranes. Most of *H. pylori* strains secrete a vacuolating protein, VacA, which strongly destabilizes the phospholipid membrane of epithelial cells [9,10]. If the liposome is very close to the bacterium, it could be expected that the release of encapsulated-drug could be done by the vacuolating effect of the protein.

This paper deals with four liposome formulations. All of them contain cholesterol because of its well-known bilayer stabilizing effect [11–13] in both biological and liposome membranes. Two different phospholipids were used: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) or epikuron 170. The main advantages of using epikuron are a cheap price and a composition containing at least 10% of phosphatidylethanolamine (PE), which was described to be a ligand for a *H. pylori* adhesin [14–16]. These two liposomal formulations (DPPC–cholesterol and epikuron–cholesterol) were compared with the same formulations but in which a synthetic glycolipid was incorporated. The glycolipid we used (Scheme 1) contains: a cholesterol group as anchor in the bilayer, four ethylene glycol units and fucose as ligand. Because of its flexibility, the ethylene glycol chain has the important function of a spacer separating the sugar (fucose) from the liposomal membrane. Some strains of *H. pylori* express an outer membrane protein (BabA2) which is able to link the fucosylated Lewis b (Le^b) histo-blood group antigen, present on human gastric epithelial cells [3,7,17,18]. In a previous study, such fucosyl neoglycolipids embedded at the surface of liposomes were shown to display good interactions with a plant lectin and a minimal destabilization of the liposomal membrane [19]. The poor stability of liposomes in the gastrointestinal tract [20] is mostly due to bile salts and pancreatic lipases [21–25]. In the stomach environment, or more generally in acidic conditions, liposomes are quite stable [24,26], thus allowing a gastric targeting.

We characterized the physico-chemical properties of the liposomes we have formulated (size, zeta potential, encapsulation efficiency), and observed by epifluorescence microscopy the interactions between fluorescent liposomes and fluorescent *H. pylori* strains.



Scheme 1. Schematic representation of the neoglycolipid used.

2. Materials and methods

2.1. Materials

The 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Ref. P0763), cholesterol (Ref. C8667), dialysis tubing cellulose membrane (Ref. D9777), sodium phosphate monobasic (Ref. S-5011) and ampicillin sodium salt (Ref. A9518) were purchased from Sigma–Aldrich. The neoglycolipids were previously synthesized in the laboratory, as already reported for the cholesteryl tetraethyleneglycol *N*-acetylglucosamine (GlcNAc-E_4-Chol) [27]. The synthesis of cholesteryl tetraethyleneglycol fucose (Fuc-E_4-Chol) was realized by methodologies already reported to prepare α-L-fucopyranosides of Guerbet alcohols [28] and will be published in due course. Fluorescent probe 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-PC, Ref. N3787) was provided by Molecular Probes™, Invitrogen. Brain Heart Infusion agar CM375, vitox SR090J (hydration fluid) and vitox SR090K (vitox supplement) were purchased from Oxoid. The metronidazole (Ref. 68035), ammonium thiocyanate (Ref. 09950) and ferric chloride hexahydrate (Ref. 44944) were purchased from Fluka. The nitrogen 2-1°, 4.5; the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI, Ref. 124653), Epikuron 170 mixture (phosphatidylcholine > 72%, phosphatidylethanolamine > 10%, phosphatidylinositol < 3%, lyso-phosphatidylcholine < 4% and free fatty acids 10%), and the DNeasy tissue kit (Ref. 69504) were provided, respectively, by Linde, Merck KGaA, Degussa, and Qiagen. All solvents and reagents were analytical grade.

2.2. Helicobacter pylori strains

Helicobacter pylori CCUG 17875 is a reference strain obtained from the Culture Collection of the University of Gothenburg. *H. pylori* 149C is a clinical strain isolated at the Istituto Cantonale di Microbiologia, Bellinzona, Switzerland.

2.3. Methods

2.3.1. Vesicle preparation

Liposomes are prepared by extrusion method [29]. Briefly, phospholipids, cholesterol and glycolipid (in a total lipid concentration of 30 mM) are dissolved in chloroform,