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Synergistic interactions between doxycycline and terpenic components of essential oils encapsulated within lipid nanocapsules against gram negative bacteria

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ABSTRACT

The combination of essential oils (EOs) with antibiotics provides a promising strategy towards combating resistant bacteria. We have selected a mixture of 3 major components extracted from EOs: carvacrol (oregano oil), eugenol (clove oil) and cinnamaldehyde (cinnamon oil). These compounds were successfully encapsulated within lipid nanocapsules (LNCs). The EOs-loaded LNCs were characterised by a noticeably high drug loading of 20% and a very small particle diameter of 114 nm. The in vitro interactions between EOs-loaded LNCs and doxycycline were examined via checkerboard titration and time-kill assay against 5 Gram-negative strains: *Acinetobacter baumannii* SAN, *A. baumannii* RCH, *Klebsiella pneumoniae*,*Escherichia coli* and *Pseudomonas aeruginosa*. No growth inhibition interactions were found between EOs-loaded LNCs and doxycycline (FIC index between 0.7 and 1.30). However, when bactericidal effects were considered, a synergistic interaction was observed (FBC index equal to 0.5) against all tested strains. A synergistic effect was also observed in time-kill assay (a difference of at least 3 log between the combination and the most active agent alone). Scanning electron microscopy (SEM) was used to visualise the changes in the bacterial membrane. The holes in bacterial envelope and leakage of cellular contents were observed in SE micrographs after exposure to the EOs–LNCs and the doxycycline combination.

1. Introduction

Antibiotic resistance is a rapidly growing problem around the world. The current emergence of multiresistant bacteria such as *Acinetobacter baumannii* represents a serious public health problem (Dennesen et al., 1998; Song et al., 2009). This is the reason why many researchers have focused their work on the search for new antibiotics by developing synthetic molecules and small-molecule libraries customised for bacterial targets. Some of them have intensified research on DNA synthesis and have studied the correlation between the chemical structure of different molecules and their biological activity (Committee on new directions in the study of antimicrobial therapeutics). Even though many effective compounds have been discovered in the past, chemical variability is insufficient to prevent an escalation of antibiotic resistance (Rosamond and Allsop, 2000). During the 2000s researchers were interested in genomes in search for new antibiotics (Rosamond and Allsop, 2000). Other researchers focused their work on derivatives of natural origin substances such as actinonin, pleuromutilin, ramoplanin and tiacumicin B (Butler and Buss, 2006) or essential oils (Hemaiswarya et al., 2008; Rosato et al., 2008, 2009; Wagner and Ulrich-Merzenich, 2009; Fadli et al., 2012). Essential oils are known to penetrate cell membranes and interact with protein targets (Stone and Williams, 1992).

We selected a mixture of three terpenic components of essential oils: carvacrol, eugenol, and cinnamaldehyde (Fig. 1).

Abbreviations: ATP, adenosine triphosphate; BHI, brain–heart infusion broth; CFU, colony forming units; EF-G, elongation factor G; EF-Tu, elongation factor thermos unstable; EOs, essential oils; FBC index, fractional bactericidal concentration index; FIC index, fractional inhibitory concentration index; HLB, hydrophilic–lipophilic balance; HMDS, hexamethyldisilazane; LNCs, lipid nanocapsules; LPS, lipopolysaccharide; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; PDI, polydispersity index; PIT, phase inversion temperature; SCIAM, service commun d'imageries et d'analyses microscopiques.

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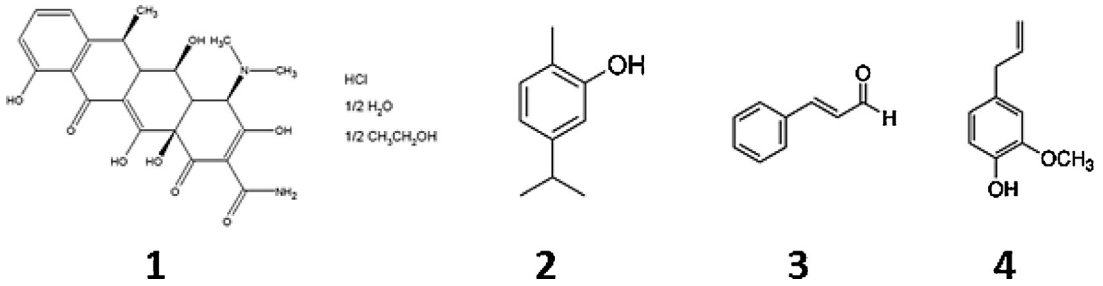

Fig. 1. Structural formulas of doxycycline hyclate (1), carvacrol (2), cinnamaldehyde (3) and eugenol (3).

These components of plant extracts exert their antibacterial activity by disrupting cell membrane and inhibiting ATPase activity (Raybaudi-Massilia et al., 2009; Negi, 2012).

Eugenol (2-methoxy-4-(2-propenyl)phenol) is a major component of clove oil (Farag et al., 1989). Eugenol is used in medicine as a local antiseptic and as an anaesthetic (Didry et al., 1994; Markowitz et al., 1992). Carvacrol (2-methyl-5-(1-methylethyl)-phenol) is a major component of oregano and thyme oil. It is also found in tequila (León-Rodríguez et al., 2008). It is an isoprenyl phenol that has strong antimicrobial activity (Roller and Sheedhar 2002) and is used in food preservation (Ben Arfa et al., 2005). *trans*-Cinnamaldehyde (3-phenyl-2-propenal) is a major organic component of cinnamon oil, used as a flavouring agent and in perfumery. Cinnamaldehyde is also used as a fungicide. It exerts hypoglycemic effect, which could be of great interest in diabetes treatment (Marles and Farnsworth, 1995; Ross, 2001)

In order to administer and to protect lipophilic substances such as essential oils, we have chosen lipid nanocapsules (LNCs) as a drug delivery system. LNCs are prepared via a phase inversion method introduced for the first time by Shinoda and Saito in 1969 (Shinoda and Saito, 1969). It is a low energy and solvent-free method based on the changes in the hydrophilic–lipophilic balance (HLB) of a polyoxyethylene surfactant caused by temperature modification (Huynh et al., 2009). This method enables the obtention of small, homogenously dispersed particles. By varying the mass ratio of the LNCs components: surfactants (a hydrophilic surfactant—Solutol®, and optionally a lipophilic surfactant-lecithin) and the oil (Labrafac®), one can yield particles with sizes ranging between 25 and 100 nm. These particles consist of a triglyceride core surrounded by a surfactant shell. One of the most important characteristics of these nanocarriers is a high surface area-to-volume ratio that can offer a considerable advantage in interactions with bacteria.

When treating infections, the use of a single antimicrobial agent is normally sufficient to achieve a desired therapeutic effect. However, there are several instances in which two or more drugs must be given together. These situations include the treatment of serious infections before the identity of the microorganism is known, a desired synergistic effect against certain organisms and the prevention of the emergence of resistant strains (Garrod, 1953; Levinson, 2014). Indeed, combined antibiotic therapy has been used against multiresistant bacteria (Song et al., 2009; Saballs et al., 2006; Yi et al., 2005; Montero et al., 2004). Owing to their action mechanism, essential oils offer a promising alternative to be used in combination with traditional antibiotics.

We have selected doxycycline (Fig. 1) tetracycline class antibiotic with bacteriostatic activity against a variety of Gram-positive and Gram-negative bacteria, mycoplasma, chlamydiae and rickettsiae. Doxycycline is used in the treatment of bacterial infections such as acne, urinary tract infections and protozoal infections such as malaria. It is the antibiotic of choice for the treatment of infections caused by *Chlamydia psittaci*, *Vibrio cholerae* (cholera), *Vibrio vulnificus*, *Mycobacterium marinum* and *Mycoplasme pneumoniae* (Bryskier, 2005). Doxycycline is useful in the treatment of respiratory tract infections because of its activity against intracellular atypical microorganisms known to be resistant to antibiotics (Brunton et al., 2006), such as β-lactams. Doxycycline has an excellent oral absorption and a satisfying half-life (Naidong et al.,1990). Its bacteriostatic activity and its rapid resistance when used alone explain why doxycycline is frequently given with other antibiotics like neomycine (Bryskier, 2005), ofloxacin, amoxicilline or antibacterial compounds like lysosomotropic agents (drugs capable of penetrating lysosomes) (Raoult et al., 1990). Moreover, in certain diseases such as endocarditis, it is often favourable to use a bactericidal drug (Finberg et al., 2004), since doxycycline cannot be used in the treatment of such infections. Other studies suggest that a combination of bactericidal and bacteriostatic agents may lead to improved clinical outcomes, compared with either agents alone (Klastersky, 1986; Chow and Yu, 1999; Finberg et al., 2004).

Considering that no doxycycline/EOs combination has been reported to date, the aim of this study was to examine in vitro interactions between essential oils encapsulated within lipid nanocapsules and doxycycline using a checkerboard method and a time-kill assay.

2. Materials and methods

2.1. Materials

Labrafac® WL1349 (caprylic/capric acid triglycerides; IVth European Pharmacopeia, 2002) was kindly provided by Gattefossé S.A. (France). Lipoid® S75-3 (hydrogenated lecithin) and Solutol® HS15 (macrogol 15 hydroxystearate, polyoxyl 15 hydroxystearate; a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) (IVth European Pharmacopeia, 2002) were kindly provided by Lipoid Gmbh (Germany) and BASF (Germany), respectively. Cinnamaldehyde and eugenol were purchased from Merck (Germany) and carvacrol from Sigma–Aldrich (UK). Doxycycline hyclate (Vibraveneuse®) (100 mg) powder for solution for intravenous infusion was furnished by Serb Laboratoires. All other chemicals and solvents were of analytical grade. Brain–heart infusion (BHI) broth was purchased from bioMérieux (France). Columbia agar supplemented with sheep blood (5%) was obtained from Oxoid (France).

2.2. Preparation of LNCs

LNCs were prepared following the procedure described by Montagu et al. (2014) with the composition presented in Table 1. The components of the LNCs (polyoxyl 15 hydroxystearate, hydrogenated lecithin, and triglycerides) and NaCl were weighed, mixed with water and heated to 90 °C. The samples were cooled to 60 °C. The samples were treated with three heating–cooling cycles, and during the last cooling cycle, at 80–90 °C (the temperature of the phase inversion) the system was diluted with cold (~4 °C)