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Rapid and soft formulation of folate-functionalized nanoparticles for the targeted delivery of tripentone in ovarian carcinoma

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ABSTRACT

We report the development of folate-functionalized nanoparticles able to target folate receptors, and to deliver a poorly water soluble cytotoxic agent, a tripentone, in ovarian carcinoma. The stability under incubation of lipid nanoparticles formulated by a low-energy phase inversion temperature method was investigated. Thanks to the presence of Labrasol®, a macrogolglyceride into the composition of the nanocarriers, the conjugation of different quantities of a folate derivate (folic acid-polyethylene glycol$_{2000}$-distearylphosphatidylethanolamine) to nanoparticles was possible by a rapid, soft, very simple post-insertion process. As determined by dynamic light scattering, nanoparticles present a monodisperse diameter of about 100 nm, a spherical shape as attested by transmission electron micrographs, a weakly negative surface zeta potential, and are able to encapsulate the tripentone MR22388. The presence of folate receptors on SKOV3 human ovarian cancer cells was identified by fluorescent immunocytochemistry. Cellular uptake studies assessed by flow cytometry indicated that these nanoparticles reached the SKOV3 cells rapidly, and were internalized by a folate-receptor mediated endocytosis pathway. Moreover, nanoparticles allowed the rapid delivery of the antitumor agent tripentone into cells as shown *in vitro* by real-time cellular activity assay. Such folate-lipid nanoparticles are a potential carrier for targeted delivery of poorly water soluble compounds into ovarian carcinoma.

1. Introduction

By many estimates 40% of *leads* discovered by the pharmaceutical industry today are poorly water-soluble drug candidates. These insolubility issues complicate their delivery, and also their bioavailability *in fine*. Different formulation strategies can be considered to enhance drug solubility, and to reduce the percentage of poorly soluble drug candidates eliminated from development. Among them, various research works report the formation of drug-cyclodextrin inclusion complexes, solid dispersions, surfactant addition, and also the development of lipid drug delivery systems (Sahoo and Labhasetwar, 2003; Seigneuric et al., 2010).

3-(3-hydroxy-4-methoxyphenyl)-8H-thieno[2,3-*b*]pyrrolizin-8-one, or MR22388, is a highly cytotoxic anti-cancer agent which can be considered as the *lead* compound of tripentone series (Lisowski et al., 2004; Rochais et al., 2013). Although its cytotoxicity mechanisms are not yet fully understood, MR22388 appears as an inhibitor of the polymerization of tubulin and a strong inhibitor of several kinases (Rochais et al., 2013). It also modulates the expression of some Bcl-2 family proteins in ovarian carcinoma cells, and could be useful for sensitization to emerging Bcl-xL targeting strategies (Tomasina et al., 2013b). Nevertheless, MR22388 presents a very poor solubility in water lower than 10 μg/mL. To overcome this lack of solubility, lipidic nanocapsules encapsulating the tripentone have been developed by using a formulation process based on an organic solvent-free low energy emulsification method (Malzert-Fréon et al., 2006),

Abbreviations: DLS, dynamic light scattering; FA-PEG-DSPE, folic acid-polyethylene glycol$_{2000}$-distearylphosphatidylethanolamine; HLD, hydrophilic/lipophilic deviation; NP, nanoparticle; o/w, oil-in-water; PDI, polydispersity index; FA, folic acid.

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and by incorporating a solubility enhancer, a caprylocaproyl macrogolglyceride (Labrasol®) (Malzert-Fréon et al., 2010).

Ovarian cancer is the leading cause of death from gynecological malignancies worldwide (Vitale et al., 2013). Although the majority of tumors initially respond to standard treatments combining surgery and chemotherapy with platinum based chemotherapy, frequent recurrence and subsequent acquired chemoresistance are responsible for the therapeutic failures, leading to an overall 5 years survival rate of 40% (Banerjee and Kaye, 2013). In this context, new drugs or novel therapeutic strategies must be found. In particular, considerable efforts are implemented to engineer systems capable of ferrying large doses of cytotoxic agents specifically into targeted malignant cells while sparing healthy cells. Various targeted colloidal systems are reported for drug delivery to ovarian tumors (Tomasina et al., 2013a).

Active site-specific targeting and effective uptake of drug nanocarriers can be achieved *via* receptors of the cell membrane, which may mediate uptake through various phagocytosis and endocytosis mechanisms (Danhier et al., 2010). The folic acid receptor constitutes a useful target for tumor specific drug delivery, especially in malignancies of the ovary (Markert et al., 2008). To activate such receptors, a recognizable cancer-specific ligand must be linked to the carrier surface. Use of folate as ligand is very attractive since it presents a high binding affinity (Kd $\sim 10^{-10}$ M), a small size (441.4 g/mol), a low immunogenicity, a good compatibility with various organic and aqueous solvents, a good stability during storage. It is easily available, and is relatively inexpensive (Lu et al., 2002).

Different strategies are reported to link ligands to nanocarriers (Zalipsky et al., 2004). Among them, one is based on a post-insertion method, *i.e.* on simple incubation of micellar ligand-polyethylene glycol (PEG)-lipid conjugates with preformed, ready-to-use, non-targeted nanocarriers. By this approach, the conjugate is exclusively positioned on the outside of the liposome bilayer (Gabizon et al., 1999; Uster et al., 1996). This post-insertion method which was initially described for liposomes, was also transposed to lipidic nanoparticles (Hoarau et al., 2004).

The objective of the present work was to render tripentone-loaded nanoparticles able to target ovarian cells, and to evaluate their cytotoxic activity on human ovarian carcinoma cells.

2. Materials and methods

2.1. Materials

Labrafac® CC (caprylic/capric acid triglycerides) and Labrasol® (caprylocaproyl macrogol-8 glycerides) were kindly provided by Gattefosse S.A. (Saint-Priest, France). Lipoid® S75-3 (soybean lecithin at 69% phosphatidylcholine and 10% phosphatidylethanolamine), and Solutol® HS-15 (70% PEG 660 hydroxystearate and 30% free PEG 660) were gifts from Lipoid GmbH (Ludwigshafen, Germany) and BASF AG (Ludwigshafen, Germany), respectively. Due to the complex composition of each product, the brand names are used throughout the text. NaCl, methanol, and acetonitrile of HPLC analytical grade were provided by Carlo Erba (Val de Reuil, France). Distilled water was obtained by an Elgastat option 3 (Elga, UK). The tripentone MR22388 (3-(3-hydroxy-4-methoxyphenyl)-8*H*-thieno[2,3-b]pyrrolizin-8-one) was provided by Syntheval (Caen, France). The fluorescent dye, Nile Red (9-(diethylamino)-5*H*-benzo[a]phenoxazin-5-one), was obtained from Invitrogen (Saint Aubin, France). Folic acid (FA), dicyclohexylcarbodiimide, DMSO, and pyridine were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France). Amino-polyethylene glycol 2000-distearoylphosphatidylethanolamine (PEG-DSPE) was provided by Avanti Polar Lipids (Alabaster, AL). SKOV3 cell line obtained from ECACC (Cerdic, Nice, France) were cultured as described (Simonin et al., 2013) with RPMI-1640 medium supplemented with 2 mM Glutamax TM, 25 mM HEPES, 10% fetal calf serum, and 33 mM sodium bicarbonate (Fisher Scientific Bioblock, Illkirch, France).

2.2. Formulation of nanoparticles (NP)

2.2.1. Unloaded NP

All components, *i.e.* Solutol® (423 mg), Labrasol® (605 mg), Labrafac® (242 mg), Lipoid® (37.5 mg), NaCl (48.6 mg) and water (1.21 g) were mixed together, and the formulation process established elsewhere was applied (Malzert-Fréon et al., 2010). Briefly, the mixture was heated under magnetic stirring (250 rpm) from room temperature to 85 °C, and then cooled down to 45 °C. This cycle was repeated twice, and then was quenched by the addition of 3 mL cold water (0 ± 1 °C) at 61 °C. The NP suspension was stirred for 5 min before use.

2.2.2. Tripentone loaded NP

In case of tripentone loaded NP, after a pre-treatment based on sonication (15 min) and heating of the compound in Labrasol® (up to 85 °C) under magnetic stirring (250 rpm), making the tripentone freely soluble in the excipient, the mixture was mixed with other compounds. The same process as the one described for blank particles was then applied.

2.2.3. Fluorescent NP

Fluorescent dye (Nile Red, 0.1 mg) was dissolved in Labrafac® under magnetic stirring (250 rpm) up to 60 °C. Other excipients were added, and NP were then prepared as described in Section 2.2.1.

2.3. Physicochemical characterization of the NP

2.3.1. Measurement of particle size and size distribution

The average hydrodynamic diameter, and the polydispersity index (PDI) of the nanoparticles were determined by dynamic light scattering (DLS) using a NanoZS apparatus (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser at a fixed scattering angle of 173°. The polydispersity index (PDI) was used as a measurement of the size distribution. A small value of PDI (<0.1) indicates a monodisperse size distribution while a PDI > 0.2 indicates a higher heterogeneity. The temperature of the cell was kept constant at 25 °C. The suspension of nanoparticles was diluted 1:11 (v/v) in distilled water in order to assure an appropriate scattered intensity on the detector, and filtrated through 0.2 μm cellulose acetate syringe filters (Fisherbrand, Fisher Scientific, Illkirch, France) before measurements.

Evolution of the diameter of NP *versus* temperature was studied by using NanoZS apparatus in the trend mode, with a heating rate of 0.5 °C/min, after a 1:11 (v/v) dilution of the suspension in distilled water.

Except for Table 1 where diameter values are given according to a distribution by volume because the size populations may be heterogeneous, all other experimental results are presented according to a size distribution by intensity.

2.3.2. Measurement of the zeta potential

Measurements were carried out using a Malvern Zetasizer NanoZS apparatus (Malvern Instruments, Worcestershire, UK) equipped with a DTS 1060 cell. A 1:11 (v/v) dilution of the suspension of nanoparticles in NaCl 1 mM was performed. Measurements were made in triplicate at 25 °C, with a dielectric constant of 78.5, a refractive index of 1.33, a viscosity of 0.8872 cP, and a cell voltage of 150 V. The zeta potential was calculated from the electrophoretic