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Solid lipid nanoparticles as intracellular drug transporters: An investigation of the uptake mechanism and pathway

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ABSTRACT

The aim of this work was to develop a systematic analysis of the cellular internalisation mechanism and pathway of solid lipid nanoparticles (SLN) internalisation. To evaluate if SLN show cell uptake and to understand the mechanism of internalisation, four human glioma cell lines (A172, U251, U373 and U87) and a human macrophage cell line (THP1) were used. For this purpose rhodamine 123 (R123) was loaded into SLN coated with polysorbate 60 and 80. Fluorescence microscopy and flow cell cytometry techniques were assessed to study internalisation of these systems within the cells. MTT studies were performed to evaluate the cytotoxicity of the R123-loaded SLN. To assess the SLN internalisation mechanism and intracellular pathway, excluding endocytosis mechanisms were applied. Our results revealed that R123-loaded SLN with mean size below 200 nm and slight negative surface charge (around −20 mV) have the ability to be internalised by gliomas in a higher amount than by macrophages. The mechanism of internalisation was found to be mainly through a clathrin-dependent endocytic pathway. In addition, the cytotoxicity of SLN was higher for gliomas than for macrophages. These results suggest that SLN can be a promising alternative in brain tumours treatment.

1. Introduction

Malignant gliomas have a very poor prognosis despite the aggressiveness of the current treatment that combines surgery, radiotherapy and chemotherapy (Westphal et al., 2003; Stupp et al., 2006). Knowing that the main obstacle to develop an effective therapy against gliomas is the difficulty of delivering anticancer drugs to the tumour site and cancer cells, several promising drug delivery systems involving nanoparticles were developed (Gelperina et al., 2010; Ying et al., 2010; Cirpanli et al., 2011; Kuo and Liang, 2011).

Solid lipid nanoparticles (SLN) (Joshi and Müller, 2009; Souto and Muller, 2010) have been reported as a promising anticancer drug delivery system to the brain after i.v. injection, due to their ability to cross the blood-brain barrier and deliver drugs to the brain, when coated with proper surfactants, such as polysorbates (Blasi et al., 2007; Brioschi et al., 2007; Kaur et al., 2008; Brioschi et al., 2009). Thus, SLN have high potential in brain tumour treatment.

SLN are colloidal particles, consisting of a matrix composed of lipids being solid at both room and body temperatures, dispersed in an aqueous surfactant solution (Joshi and Müller, 2009). SLN combine advantages of other colloidal drug delivery systems such as emulsions, liposomes and polymeric nanoparticles, and at the same time avoid or minimise some of their drawbacks. Some of the advantages of SLN are the ability to immobilise hydrophilic or hydrophobic drugs in the solid matrix and sustain the drug release, and the ability to prevent the premature degradation of the incorporated drug. Another advantage of the use of lipid particles as drug carrier systems is the fact that the matrix is composed of physiological components and/or excipients of accepted status (FDA-approved constituents), decreasing the risk of acute and chronic toxicity (Joshi and Müller, 2009; Souto and Muller, 2010).

After crossing the blood-brain barrier the next important question is whether the SLN can be internalised by the tumour cell and release anticancer drugs inside them. Nanoparticles located in the external environment of a cell can interact with the plasma membrane, which can lead to the uptake of these nanoparticles

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; PMA, propidium iodide, phorbol 12-myristate 13-acetate; R123, rhodamine 123; SLN, solid lipid nanoparticles.

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by the cells through a process named "endocytosis" (Sahay et al., 2010). If the nanoparticles cannot be internalised, the drug can still enter cells after being released from the nanoparticles but the drug can also disperse to the surrounding normal tissues rather than be delivered mainly to the cancer cells. In fact, *in vitro* and *in vivo* studies reveal that the intracellular concentration of the drug is much higher when it is released from nanoparticles into the cytoplasm after internalisation (Sugano et al., 2000; Chen, 2010; Jain et al., 2010).

The form of endocytosis involved in nanoparticles uptake can be expected to affect the nanoparticle's intracellular localisation and trafficking. Understanding endocytic mechanisms is then crucial for the development of nanoparticles for clinical therapies.

Furthermore, most nanoparticles have been shown to exploit more than one pathway to gain cellular entry (Sahay et al., 2010). The endocytosis of nanoparticles also depends on the cell type treated (Lai et al., 2007; Thurn et al., 2007).

Bearing in mind that cell type could be critical in defining the nanoparticle entry and final destination in the cells, we selected four human glioma cell lines (A172, U251, U373 and U87) and one human monocytic cell line (THP1) to study the uptake mechanism of SLN by human glioma cells and macrophages.

Endocytosis is known as a general entry mechanism for various extracellular materials and can be divided into two main categories: phagocytosis (uptake of large particles) and pinocytosis (uptake of fluids and solutes) (Doherty and McMahon, 2009; Sahay et al., 2010). Phagocytosis is followed by specialised professional phagocytes, such as macrophages, monocytes, or dendritic cells. The phagocytic pathway of cellular entry consists of recognising the particles followed by the adhesion of the opsonised particles onto the cell membrane and ingestion of the particle by the cells.

Pinocytosis, in contrast, is present in all types of cells and has multiple forms depending on the cell origin and function. Pinocytosis can be classified as clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin- and caveolae-independent endocytosys, and macropinocytosis (Conner and Schmid, 2003).

Internalisation through clathrin-dependent endocytosis happens when the clathrin coat on the plasma membrane develops invaginations in the membrane leading to the budding of clathrin-coated vesicles (Sahay et al., 2010). Nanoparticles localised on the cell membrane could be trapped within the vesicles and brought within the cells. Receptor-mediated endocytosis through clathrin-coated pits is the most common pathway of endocytosis. Alternatively, clathrin-independent endocytosis can happen through the caveolae or lipid-raft pathway. Caveolae are flask-shaped membrane invaginations on cell surfaces that have high amounts of cholesterol and sphingomyelin. Caveolae are abundant in muscle, endothelial cells, fibroblasts and adipocytes and absent in neurons and leukocytes (Conner and Schmid, 2003; Sahay et al., 2010). In the macropinocytosis, the macropinosomes are larger (0.5–10 μm) and distinct from other vesicles formed during pinocytosis. This pathway is possible for virtually any cell with only a few exceptions, such as macrophages and brain microvessel endothelial cells. At first glance, it can internalise large particles with submicron and greater sizes in cells, which lack phagocytosis (Sahay et al., 2010).

To clarify the endocytosis mechanism of nanoparticles internalisation, specific endocytosis mechanisms can be excluded by using pharmacologic inhibitors (Kam et al., 2006; Thurn et al., 2011). Endocytosis is known as a general entry mechanism for various extracellular materials and it is an energy dependent uptake. Consequentially it is inhibited when incubations are carried out at low temperature (e.g. 4 °C instead of 37 °C) (Kam et al., 2006; Thurn et al., 2011). Furthermore, to assess the role of clathrin in the internalisation of SLN, incubations under hypertonic environments (e.g. sucrose 0.45 M) that are recognised to disrupt the formation of clathrin-coated vesicles on the cell membrane could be carried out (Kam et al., 2006; Sahay et al., 2010). To evaluate SLN cellular uptake through the caveolae or lipid-rafts pathway, cells could be pretreated with the drug filipin, which is known to disrupt the cholesterol distribution within the cell membrane (Kam et al., 2006; Sahay et al., 2010). To assess if the cellular entry occurs by macropinocytosis (gliomas) or phagocytosis (macrophages) the cells could be pretreated with cytochalasin B, a potent inhibitor of macropinocytosis/phagocytosis, which depolymerises the actin filaments avoiding the formation of the structures essential to enclose particulates (Serda et al., 2009).

The cells with excluded endocytosis mechanisms were studied along with the 5 cell lines by flow cell cytometry allowing determination of the uptake pathway of the SLN. Furthermore, fluorescence microscopy was performed to visualise SLN uptake and distribution within the cells.

The aim of this work was to develop a systematic analysis of the cellular internalisation mechanism and pathway for SLN to understand the mechanisms behind this internalisation.

2. Materials and methods

2.1. Materials

The wax cetyl palmitate was a gift from Gattefossé SA (France). The surfactants polysorbate 60 and 80 were provided by Merck (KgaA, Germany). Rhodamine 123 (R123), thiazolyl blue tetrazolium bromide (MTT assay), propidium iodide, phorbol 12-myristate 13-acetate (PMA), collagen from rat tail, filipin III from *Streptomyces filipinensis* (Filipin) and cytochalasin B were obtained from Sigma–Aldrich (Portugal). 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) and Alexa Fluor® 594 conjugated of wheat germ agglutinin (Alexa) were obtained from Molecular probes (USA). D(+)-Sucrose was purchased from Romil Pure Chemistry (UK). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), glutamine, penicillin-streptomycin, Fungizone, RPMI Medium 1640 and Hanks' balanced salt solution (HBSS) were provided by Gibco (Invitrogen Corporation, Spain). Dimethyl sulfoxide (DMSO) was obtained from Merck (KgaA, Germany). Purified water was of MilliQ®-quality.

2.2. Annotation for formulation

Unique codes were selected for identifying the produced formulations; it consists of abbreviations for the fluorescent probe R123; the lipid cetyl palmitate (CP) and the surfactants polysorbate 60 (P60) and polysorbate 80 (P80). CP60 and CP80 stand for unloaded cetyl palmitate-based SLN stabilised with polysorbate 60 or polysorbate 80; respectively. R123-CP60 and R123-CP80 stand for rhodamine 123 incorporated into cetyl palmitate-based SLN stabilised with polysorbate 60 or polysorbate 80; respectively.

2.3. Methods

2.3.1. Production of solid lipid nanoparticles

Formulations containing the lipid cetyl palmitate and the surfactants polysorbate 60 or 80, were prepared at concentrations of 5% (w/w) of lipid and 2% (w/w) of surfactant. One lipid and one surfactant were combined at a time. SLN were prepared by the high shear homogenisation and ultrasonication techniques. Briefly, the lipid and surfactant mixture was melted at approximately 5–10 °C above the melting point of the lipid. Water was heated at approximately the same temperature and transferred to the surfactant