International Journal of Pharmaceutics 487 (2015) 205–212

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

In vitro and *in vivo* evaluation of Δ^9-tetrahidrocannabinol/PLGA nanoparticles for cancer chemotherapy

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ARTICLE INFO

Article history:
Received 24 February 2015
Received in revised form 15 April 2015
Accepted 16 April 2015
Available online 18 April 2015

Keywords:
Blood compatibility
Chitosan
Δ^9-Tetrahidrocannabinol
Lung cancer
Nanoparticles
PEGylation
PLGA

ABSTRACT

Nanoplatforms can optimize the efficacy and safety of chemotherapy, and thus cancer therapy. However, new approaches are encouraged in developing new nanomedicines against malignant cells. In this work, a reproducible methodology is described to prepare Δ^9-tetrahidrocannabinol (Δ^9-THC)-loaded poly(D,L-lactide-*co*-glycolide) (PLGA) nanoparticles against lung cancer. The nanoformulation is further improved by surface functionalization with the biodegradable polymers chitosan and poly(ethylene glycol) (PEG) in order to optimize the biological fate and antitumor effect. Mean nanoparticle size (≈290 nm) increased upon coating with PEG, CS, and PEG–CS up to ≈590 nm, ≈745 nm, and ≈790 nm, respectively. Surface electrical charge was controlled by the type of polymeric coating onto the PLGA particles. Drug entrapment efficiencies (≈95%) were not affected by any of the polymeric coatings. On the opposite, the characteristic sustained (biphasic) Δ^9-THC release from the particles can be accelerated or slowed down when using PEG or chitosan, respectively. Blood compatibility studies demonstrated the adequate *in vivo* safety margin of all of the PLGA-based nanoformulations, while protein adsorption investigations postulated the protective role of PEGylation against opsonization and plasma clearance. Cell viability studies comparing the activity of the nanoformulations against human A-549 and murine LL2 lung adenocarcinoma cells, and human embryo lung fibroblastic MRC-5 cells revealed a statistically significant selective cytotoxic effect toward the lung cancer cell lines. In addition, cytotoxicity assays in A-549 cells demonstrated the more intense anticancer activity of Δ^9-THC-loaded PEGylated PLGA nanoparticles. These promising results were confirmed by *in vivo* studies in LL2 lung tumor-bearing immunocompetent C57BL/6 mice.

1. Introduction

Δ^9-Tetrahidrocannabinol (Δ^9-THC) has attracted special interest in oncology given their well-known palliative effects and antitumor activity (Ramer and Hinz, 2008; Aviello et al., 2012; Velasco et al., 2012; Solinas et al., 2013). In fact, Δ^9-THC has been described to inhibit tumor angiogenesis and cell growth in malignant tissues, thus causing cell death (McKallip et al., 2002; Casanova et al., 2003; Blázquez et al., 2004; Bifulco et al., 2006; Ramer et al., 2012; Hernán Pérez de la Ossa et al., 2013a; Machado-Rocha et al., 2014).

Unfortunately, and despite oral aerosols, transdermal patches, and suppositories have been proposed (Hernán Pérez de la Ossa et al., 2013a), up to now the development of an effective and safety (Δ^9-THC)-based formulation remains to be accomplished. Probably, reasons beneath the challenge are the high instability, oily-resin nature, low water solubility (≈2.8 mg/L), and low bioavailability of the compound (Brownjohn and Ashton, 2012). To beat the challenge, (Δ^9-THC)-loaded microparticulate systems based on the polymer poly(ε-caprolactone) have been engineered by oil-in-water emulsion-solvent evaporation with promising results against cancer (Hernán Pérez de la Ossa et al., 2012,

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http://dx.doi.org/10.1016/j.ijpharm.2015.04.054
0378-5173/

2013a,b). However, the micrometer size of the Δ^9-THC particulate formulation (≈20–50 μm) is expected to limit the clinical outcome (Decuzzi et al., 2009).

As an alternative, poly(D,L-lactide-*co*-glycolide) nanoparticles (PLGA NPs) have been investigated (Martín-Banderas et al., 2014). This PLGA-based formulation was also used as nanocarrier for synthetic cannabinoid receptor agonist 13 (CB13) molecules (Durán-Lobato et al., 2013; Martín-Banderas et al., 2012). However, a definitive *in vitro* and *in vivo* proof of concept of the possibilities of this (Δ^9-THC)-loaded nanoparticulate formulation is needed.

Therefore, this work is devoted to the development of PLGA-based nanocarriers as delivery systems for Δ^9-THC. Concretely, PLGA NPs, PLGA NPs surface coated with poly(ethylene glycol) (PEGylated PLGA NPs), PLGA NPs embedded within a chitosan (CS) shell (chitosan-coated PLGA NPs), and PEGylated chitosan-coated PLGA NPs were investigated. Vitamin E molecules were incorporated to the formulations to enhance the stability of Δ^9-THC against oxidation. Geometry and surface electrical charge measurements, blood compatibility and protein adsorption characterizations, and the *in vitro* evaluation of the Δ^9-THC loading and release capabilities revealed that the PEGylated PLGA nanosystem was the more adequate formulation for the parenteral administration of Δ^9-THC (see below). Finally, the *in vitro* anticancer activities of (Δ^9-THC)-loaded PEGylated PLGA NPs were evaluated in murine LL2 and human A-549 lung cancer cell lines. The human embryo lung fibroblastic MRC-5 cell line was used as control. Regarding the *in vivo* investigation of the antitumor potential of (Δ^9-THC)-loaded PEGylated PLGA NPs, LL2 lung tumor-bearing immunocompetent C57BL/6 mice was used to that aim.

2. Materials and methods

2.1. Materials

Δ^9-THC was provided by THC Pharm (Frankfurt am Main, Germany). PLGA (Resomer® RG 502, PLGA 50:50, molecular weight: 12,000 Da, inherent viscosity: 0.24 dL/g) was obtained from Boehringer-Ingelheim (Ingelheim, Germany). Low molecular weight chitosan, Span® 60, Tween® 80, Pluronic® F-68, ethylenediaminetetraacetic acid (EDTA), trypsin, sodium citrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton® X-100, ammonium oxalate, sodium dodecyl sulphate (SDS), monoclonal antibody specific for human C3a desArg peroxidase-conjugated rabbit anti-C3a and fluorochrome nile red were purchased from Sigma–Aldrich (St Louis, MO, USA). Glycerol was obtained from Acofarma Distribución S.A. (Barcelona, Spain). Vitamin E was a generously gift from ChemTrade GmbH (Burgbernheim, Germany). Threalose was obtained from VWR International Eurolab S.L. (Barcelona, Spain).

PEG (molecular weight: 20 kDa) was obtained from Serva Feinbiochemica GmbH & Co. (Heidelberg, Germany). PEG molecular weights and PEG chain lengths may have a significant influence on the prevention of the surface adsorption of plasma proteins onto NPs (and thus, plasma clearance) (Gref et al., 2000). It is accepted that increasing PEG chain lengths (thus PEG molecular weights) minimizes protein adsorption onto PEGylated NPs (preventing plasma clearance). Hence, this is the reason why PEG with a molecular weight of 20 kDa was used, instead of other PEGs, *e.g.*, from 2000 to 5000 Da.

All other chemicals were purchased from Panreac Química (Barcelona, Spain). Deionized and filtered water was used in all the experiments (Milli-Q Academic, Millipore, Molsheim, France). The release medium (phosphate buffered saline, PBS, pH 7.4) was obtained from Biochemica AppliChem (Darmstadt, Germany).

Human embryo lung fibroblastic MRC-5 and human lung adenocarcinoma A-549 cell lines were obtained from the European Collection of Cell Cultures (ECACC). The murine lung carcinoma LL2 cell line was obtained from the Scientific Instrumentation Centre of the University of Granada (Granada, Spain). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM high glucose, with L-glutamine, phenol red and without pyruvate) with penicillin–streptomycin, which were obtained from PAA Laboratories (Pasching, Austria), and supplemented with Fetal Bovine Serum (FBS, Stemcell Technologies Inc.).

2.2. Synthesis and characterization of the PLGA-based nanoformulations

PLGA-based NPs were prepared by following the nanoprecipitation method (Fessi et al., 1989; Martín-Banderas et al., 2014). Briefly, a weighted amount of PLGA was dissolved with Span® 60 in acetone to obtain a 1.5% (w/v) concentration. Then, 5 mL of such solution were added drop-wise to 15 mL of a Pluronic® F-68 aqueous solution (0.5%, w/v) under magnetic stirring. Acetone was evaporated at room temperature during 4 h, to obtain an aqueous dispersion which was finally centrifuged to collect the NPs (10,000 rpm, 15 min, 4 °C; Eppendorf 504R, Eppendorf AG, Hamburgo, Germany). After washing twice, the NPs were re-suspended in a 5% (w/v) threalose solution (used as cryoprotectant) and freeze-dried (frozen in liquid nitrogen and lyophilized, −80.0 ± 0.5 °C, 0.057 mbar; Telstar Cryodos-50; Telstar Industrial S. L., Tarrasa, Spain) to obtain a fine whitish powder. All the formulations were prepared in triplicate.

Surface modification of the PLGA NPs with chitosan and/or PEG was done to improve the NP cellular uptake and to minimize the opsonization process that may take place in blood (Gref et al., 2000; Parveen and Sahoo, 2011). To that aim, chitosan or PEG was added to the NP aqueous dispersion upon evaporating acetone. Briefly, PLGA NPs were incubated overnight in a 0.25% (w/v) chitosan solution in 1% (v/v) acetic acid to obtain chitosan-coated PLGA NPs. PEGylation of the chitosan-coated PLGA NPs (or PLGA NPs) was accomplished by incubating the particles in a 4.5% (w/v) PEG solution during 3 h. Finally, the PEGylated chitosan-coated PLGA NPs (or PEGylated PLGA NPs) were collected by centrifugation (10,000 rpm, 15 min, 4 °C).

To sum up, 4 nanoformulations were synthesized, *i.e.*, PLGA NPs, chitosan-coated PLGA NPs (CS–PLGA NPs), PEGylated PLGA NPs (PEG–PLGA NPs), and PEGylated chitosan-coated PLGA NPs (PEG–CS–PLGA NPs).

On the basis of the best drug incorporation conditions defined previously (Martín-Banderas et al., 2014), Δ^9-THC-PLGA was loaded to the nanoparticulate systems. Briefly, the procedure started by adding 2.25 mL of a Δ^9-THC solution in acetone (1 mg/mL) to the PLGA organic solution before starting the nanoprecipitation procedure. The drug/polymer concentration in this solution was 5% (w/w). In addition, the antioxidant additive vitamin E was incorporated to that organic solution to prevent Δ^9-THC oxidation (vitamin/polymer concentration: 5%, w/w).

Mean particle size was determined at 25.0 ± 0.5 °C by a laser scattering technique based on the Mie theory (Partica LA-950V2, Horiba Ltd., Kyoto, Japan). Measurements of aqueous dispersions of the PLGA-based NPs (0.1%, w/v) were carried out under continuous magnetic agitation. To confirm the results transmission electron microscopy (TEM) characterizations were also done (CM-10 transmission electron microscope; Philips Electronics, Amsterdam, Holland) to particle samples prepared by drying the dispersions at room temperature overnight in a convection oven. Finally, the surface electrical charge of the NPs was determined in triplicate by zeta potential (ζ, mV) measurements (Zetamaster 3000, Malvern Instruments Ltd., Malvern, UK) at room temperature.