European Journal of Pharmaceutical Sciences 99 (2017) 185–192

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

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

Development of novel self-assembled ES-PLGA hybrid nanoparticles for improving oral absorption of doxorubicin hydrochloride by P-gp inhibition: *In vitro* and *in vivo* evaluation

Jia Wang [a], Lin Li [a], Lei Wu [b], Bingjun Sun [a], Yuqian Du [a], Jin Sun [a,c], Yongjun Wang [a], Qiang Fu [a], Peng Zhang [b,*], Zhonggui He [a,*]

[a] Department of Pharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University, No. 103 Wenhua Road, Shenyang 110016, China
[b] Department of Pharmaceutical Analysis, School of Pharmacy, Shenyang Pharmaceutical University, No. 103Wenhua Road, Shenyang 110016, China
[c] Municipal Key Laboratory of Biopharmaceutics, Shenyang Pharmaceutical University, Shenyang 110016, China

ARTICLE INFO

Article history:
Received 7 September 2016
Received in revised form 6 November 2016
Accepted 14 December 2016
Available online 15 December 2016

Keywords:
Doxorubicin hydrochloride
Enoxaparin sodium
Electrostatic interaction
Self-assembled ES-PLGA nanoparticles
Oral bioavailability

ABSTRACT

To increase the encapsulation efficiency and oral absorption of doxorubicin hydrochloride (DOX), a novel drug delivery system of enoxaparin sodium-PLGA hybrid nanoparticles (EPNs) was successfully designed. By introducing the negative polymer of enoxaparin sodium (ES) to form an electrostatic complex with the cationic drug, DOX, the encapsulation efficiency (93.78%) of DOX was significantly improved. The X-ray diffraction (XRD) results revealed that the DOX-ES complex was in an amorphous form. An *in vitro* release (pH 6.8 PBS) study showed the excellent sustained-release characteristics of DOX-loaded EPNs (DOX-EPNs). In addition, *in situ* intestinal perfusion and intestinal biodistribution experiments demonstrated the improved membrane permeability and intestinal wall bioadhesion of DOX-EPNs, and caveolin- and clathrin-mediated endocytosis pathways were the main mechanisms responsible. The cytotoxicity of DOX was significantly increased by EPNs in Caco-2 cells, compared with DOX-Sol. Confocal laser scanning microscope (CLSM) images confirmed that the amount of DOX-EPNs internalized by Caco-2 cells was higher than that of DOX-Sol showing that P-glycoprotein-mediated drug efflux was reduced by the introduction of EPNs. The qualitative detection of transcytosis demonstrated the ability of the nanoparticles (NPs) to cross Caco-2 cell monolayers. An *in vivo* toxicity experiment demonstrated that DOX-EPNs reduced cardiac and renal toxic effects and were biocompatible. An *in vivo* pharmacokinetics study showed that the $AUC_{(0\text{-}t)}$ and $t_{1/2}$ of DOX-EPNs were increased to 3.63–fold and 2.47–fold in comparison with DOX solution (DOX-Sol), respectively. All these results indicated that the novel EPNs were an excellent platform to improve the encapsulation efficiency of an aqueous solution of this antitumor drug and its oral bioavailability.

1. Introduction

In many parts of the world, cancer is a major public health problem (Siegel et al., 2012). Chemotherapy, in which antitumor drugs are administered by intravenous injection, is a powerful and very popular treatment. However, it is less than ideal because of the systemic toxicity (Alibolandi et al., 2015) and administration route. Oral chemotherapy is a preferred therapeutic method because of the advantages of easy application and good patient compliance (Cho et al., 2014; Mei et al., 2013). However, oral chemotherapy has a number of problems such as low oral bioavailability (DeMario and Ratain, 1998), low permeability in the gastrointestinal tract and cytotoxic drug efflux mediated by P-glycoprotein (Luo et al., 2014). Accordingly, the development of a novel oral delivery system to increase the oral bioavailability of anticancer agents and reduce their systemic toxicity is urgently needed.

Doxorubicin hydrochloride (DOX) belongs to the anthracycline family of DNA-intercalating agents (Hortobágyi, 1997) with the important ability to unite with DNAs and suppress nucleic acid synthesis (Lee et al., 2002; Perez-Arnaiz et al., 2014), and it is a broad-spectrum antitumor drug (Ahmed et al., 2006; Wang et al., 2011). Unfortunately, DOX is far from ideal for oral administration treatment on account of its low oral bioavailability accompanied by its high affinity for the P-gp pump, low intestinal permeability and rapid hydrolysis in the stomach (Wang et al., 2015).

Poly (lactic-*co*-glycolic acid) is a biodegradable polymer which is approved by the USFDA and European Medicine Agency (EMA) for human use (Danhier et al., 2012). As pharmaceutical carriers, PLGA NPs possess a P-gp inhibition effect and multidrug resistance (MDR) reversal activity (Sahoo and Labhasetwar, 2005) and the encapsulation of antitumor

* Corresponding authors at: Mailbox 59#, School of Pharmacy, Shenyang Pharmaceutical University, No. 103 Wenhua Road, Shenyang 110016, China.
E-mail addresses: zhangpengspu@163.com (P. Zhang), hezhonggui@vip.163.com (Z. He).

http://dx.doi.org/10.1016/j.ejps.2016.12.014
0928-0987/

drugs into PLGA nanoparticles may be a good method to increase oral bioavailability. Despite the fact that many hydrophobic drugs can be incorporated easily into PLGA nanoparticles, the encapsulation of water-soluble ionic drugs may be a problem with common preparation methods (Bilati et al., 2005; Dillen et al., 2006; Saxena et al., 2004; Tewes et al., 2007), which can involve complicated processes and relatively low encapsulation efficiency.

In our design, enoxaparin sodium (ES), a type of low molecular weight heparin sodium, was introduced as a counter ion polymer to produce an electrostatic interaction with the cationic drug DOX. The complex was encapsulated into PLGA by charge neutralization. The effect of the electrostatic interaction between DOX and ES on encapsulation efficiency was illustrated using different ratios of ES/DOX. The prepared DOX-EPNs were characterized in terms of their size, morphology, DSC, stability and *in vitro* release. *In situ* single-pass intestinal perfusion was used to study the intestinal permeability of DOX-EPNs and the intestinal biodistribution of the formulations was monitored using an *in vivo* imaging system. Everted intestinal rings were used to study the endocytosis mechanisms of EPNs while the cytotoxicity of DOX-EPNs was investigated by MTT assay in Caco-2 cells. To clarify whether the EPNs had a P-gp inhibition effect, the fluorescence of EPNs accumulated in Caco-2 cells was compared with that obtained with the P-gp inhibitor verapamil (VRP). The transport of NPs across Caco-2 monolayers was also studied followed by a toxicity assay and *in vivo* pharmacokinetic study in rats finally.

2. Materials and Methods

2.1. Materials

Doxorubicin hydrochloride (DOX·HCl) was obtained from Dalian Meilun Biological Technology Co., Ltd. (Dalian, China). Poly (DL-lactic-*co*-acid) (PLGA, 75:25, Mw 10,000) was purchased from Shangdong Institute of Medical Instrument (Jinan, China). 1.2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly-ethylene glycol)-2000](PEG-DSPE) was obtained from Shanghai Advanced Vehicle Technology Co., Ltd. (Shanghai, China). Enoxaparin sodium (ES, Mw 4070) was purchased from Hebei Changshan Biochemical Pharmaceutical Co., Ltd. (Hebei, China). PVA (Mw 20,000– 30,000, degree of alcoholysis 88%) was purchased from ACROS (New Jersey, USA). MEMEBSS was acquired from Hyclone (Logan, US). Fetal bovine serum was acquired from Gibco (BRL, MD, USA). Rhodamine-labeled phalloidin, 4′, 6-diamidino-2-phenylindole (DAPI) and DiR were purchased from Fanbo Biochemical (Beijing, China). SOD and MDA assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Caco-2 cell lines were purchased from the American Tissue Culture Collection (USA). All the solvents used in this study were of HPLC grade.

2.2. Preparation and Characterization of the DOX-ES Complex

The DOX-ES complex was prepared by the co-precipitation method (Li et al., 2006). Aliquots of an ES solution were added to a DOX solution slowly under constant stirring at room temperature to obtain the required ionic molar ratio of ES to DOX. The precipitate was collected by centrifugation, washed several times with deionized water and then lyophilized for 24 h.

X-ray diffraction studies of different samples were conducted with a D/Max 2500 PC X-ray diffractometer (Rigaku, Japan) at room temperature. The powder samples were scanned from 5° to 50° (2θ) at a voltage of 50 kV.

2.3. Preparation of DOX-EPNs

DOX-EPNs were prepared by the emulsion-solvent evaporation method. Briefly, 20 mg PLGA polymer was dissolved in 1 mL dichloromethane to give a concentration of 20 mg/mL, and PEG-DSPE with a weight ratio of 10% relative to PLGA polymer was dissolved in 1%PVA aqueous solution. Then, a DOX (with a weight ratio of 10% relative to PLGA) and an ES (with a charge ratio of 1:1 to DOX) aqueous solution was added dropwise to the dichloromethane under magnetic stirring. The mixture was then added to 5 mL aqueous solution and, following ultrasonic using a probe sonicator for 5 min and subsequent solvent evaporation at room temperature, the final concentration of DOX in the nanoparticles suspension was 0.4 mg/mL. To verify the effect of ES in increasing the encapsulation efficiency, PLGA nanoparticles (PLGANPs) without ES were prepared by the same method except that the ES solution was replaced by distilled water.

2.4. Characterization of DOX-EPNs

2.4.1. Size Distribution

The size and polydispersity index of the prepared DOX-EPNs were measured using a Zetasizer (Nano ZS, Malvern Co., U.K.) and the measurements were carried out in triplicate.

2.4.2. Morphology of DOX-EPNs

The diluted DOX-EPNs solution was dropped onto a carbon-coated copper grid then, following negative staining with 2% (*w*/*v*) phosphotungstic acid, the morphology of the DOX-EPNs was examined by transmission electron microscopy (TEM, JEM-2100, JEOL, Japan).

2.4.3. Differential Scanning Calorimetry (DSC)

DSC analysis of samples was conducted using a DSC1 (Mettler Toledo, Switzerland). Samples, including DOX, blank excipients, a physical mixture of DOX and blank excipients, and lyophilized DOX-EPNs, were analyzed over the range 30–300 °C at a rate of 10 °C/min.

2.4.4. Stability Study

A Zetasizer was used to confirm the stability of DOX-EPNs. An aliquot of 100 μL DOX-EPNs was diluted with 1 mL simulated gastric fluid (SGF) or simulated intestinal fluid (SIF), and then the particle size and polydispersity index were measured at defined intervals.

2.4.5. In Vitro Release

The *in vitro* release study of DOX from DOX-Sol and DOX-EPNs was conducted by dialysis at 37 °C in phosphate buffer (PBS, pH 6.8). A liquots of 2 mL DOX-Sol or DOX-EPNs were sealed in dialysis bags (MWCO = 14 kDa) and immersed in 30 mL release medium at 37 °C under constant shaking (100 rpm). At designated times, 1 mL aliquots of the samples were removed from the flask, and replaced with the same volume of fresh medium and then high performance liquid chromatography (HPLC, Waters, USA) was carried out at 258 nm.

2.4.6. Drug Encapsulation Efficiency

The encapsulation efficiency of DOX in DOX-EPNs was determined by gel column chromatography. Drug-loaded nanoparticles suspension was loaded into a Sephadex G50 column and eluted with distilled water. DOX-EPNs were then collected and mixed with a suitable quantity of acetonitrile/0.1 M HCl (3:1, v/v). After sonication for 5 min to collapse the NPs, the volume was metered to 10 mL and the samples were analyzed by HPLC. Chromatographic separation was performed on a Diamonsil C_{18} (200 mm × 4.6 mm, 5 μm; Dikma Technologies, China) using methanol-0.01 mol/L ammonium dihydrogen phosphate- acetic acid (62:38:0.5, *v*/v/v) as mobile phase at detection wavelength of 258 nm.

2.5. Animals

Kunming mice (Male, 18–22 g) and Sprague-Dawley (Male, SD, 220–250 g) rats were obtained from the Laboratory Animal Center of Shenyang Pharmaceutical University (Shenyang, Liaoning, China). All animal experiments in this study were executed according to the