European Journal of Pharmaceutical Sciences 93 (2016) 141–146

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

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

Inulin based micelles loaded with curcumin or celecoxib with effective anti-angiogenic activity

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ARTICLE INFO

Article history:
Received 25 May 2016
Received in revised form 9 August 2016
Accepted 13 August 2016
Available online 15 August 2016

Chemical compounds studied in this article:
Inulin (PubChem CID: 16219508)
Vitamin E (PubChem CID: 14985)
Vitamin E Succinate (PubChem CID: 20353)
Celecoxib (PubChem CID: 2662)
Curcumin (PubChem CID: 969516)

ABSTRACT

Curcumin (CUR) and celecoxib (CLX) are two highly hydrophobic drugs which show bioavailability problems due to their poor aqueous solubility. The aim of this study was to encapsulate each of these drugs in micelles based on biodegradable and amphiphilic polymers to investigate their anti-angiogenesis activity. Here we use an amphiphilic polymer, based on two natural substances from renewable resources (Inulin and Vitamin E, INVITE), as a self-assembling system for the drug delivery of CUR and CLX. By the *in vivo* assay of chick embryo chorioallantoic membrane (CAM) it was assessed that both INVITE-CUR and INVITE-CLX micelles possess remarkable anti-angiogenic activity, while the INVITE micelles alone resulted intrinsically pro-angiogenic. Furthermore, it has been shown that encapsulation of CUR and CLX in INVITE micelles enhances of several magnitudes the water-solubility of CUR and CLX ($14 \cdot 10^5$ and $3 \cdot 10^2$ times for CUR and CLX, respectively).
These results may have interesting implications not only in anticancer or diabetic maculopathy therapy based on the anti-angiogenesis strategy but also for regenerative medicine where over-production of new vessels is required.

Keywords:
Angiogenesis
Micelle
Inulin
Vitamin E
Celecoxib
Curcumin

1. Introduction

Angiogenesis, *i.e.,* the formation of new blood vessels, is a crucial process in physiological and pathological conditions (Bikfalvi et al., 2011). In particular, angiogenesis is essential for the development of cancer diseases (Potente et al., 2011) being an appropriate network of capillaries necessary for tumor growth, invasion, and metastasis (Gacche and Meshram, 2014). Thus, inhibition of angiogenesis may represent an important approach in anticancer therapy and the current status of such anti-angiogenesis-based strategy for cancer treatment has recently been reviewed (Gacche and Meshram, 2014). In this context, it has been pointed out that, to overcome the side effects observed with the anti-angiogenic therapy including bleeding, thrombosis, lymphopenia, and immunomodulation, novel delivery approaches are required (Cao, 2016; Gacche and Meshram, 2014). More specifically, to address these limitations, it has been suggested that the development of targeted drug delivery systems (DDS), able to discriminate between the tumor cells and the healthy ones, may have a great potential. Thus, in recent years increasing interest there has been in using therapeutic systems based on nanotechnology approaches for targeted delivery of anti-angiogenic agents. To date, there are about thirteen FDA approved anti-angiogenic drugs ranging from macromolecular structures (*e.g.*, monoclonal antibodies) to small-molecule drugs (*e.g.,* tyrosine kinase inhibitors) (Cao, 2016; Gacche and Meshram, 2014). Most of these therapeutic agents may undergo enzymatic and chemical degradation in physiological environment or show other physicochemical unfavorable properties. Besides passive or active targeting to tumor site, encapsulation in colloidal DDS of anti-angiogenic drugs may provide several additional advantages

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http://dx.doi.org/10.1016/j.ejps.2016.08.027
0928-0987/

compared to conventional delivery, including protection of the drug from degradation and controlled release. In the recent past, polymeric nanoparticles have been mainly used for the delivery of anti-angiogenic agents, even though lipid-based nanovectors (*e.g.,* liposomes) have also been investigated (Banerjee et al., 2011).

In this context, our attention was focused on the anti-angiogenic activity of two hydrophobic drugs curcumin (CUR) and celecoxib (CLX) which show bioavailability problems due to their poor aqueous solubility limiting so their clinical application (Riikonen et al., 2015; Salem et al., 2014; Trapani et al., 2004). CLX, a potent COX-2 inhibitor used for the treatment of rheumatoid arthritis and osteoarthritis (Kismet et al., 2004), exerts also an antitumor activity dependent on its anti-angiogenic activity (Gately and Li, 2004). Furthermore, several evidence, have demonstrated an anti-angiogenic activity of CUR in tumor growth (Bimonte et al., 2015; Fu et al., 2015).

Among the nanotechnology approaches for targeted delivery of hydrophobic drugs, polymeric micelles showed an interesting potential due to their ability to encapsulate poorly water-soluble drugs inside the hydrophobic core. Polymeric micelles, indeed, consist of a hydrophilic shell enclosing a hydrophobic core, and they are generally prepared starting from an amphiphilic copolymer with self-assembling properties in water. Moreover, their small size (often ranging from 1 to 100 nm) enables to achieve passive targeting by their extravasation through the leaky tumor vessels *via* the EPR effect.

It should be noted that, there are few examples of nanosystems based on polymeric micelles loaded with CUR showing their potential in inducing anti-angiogenic effects (Lv et al., 2014; Wang et al., 2013; Yang et al., 2015). Moreover, the polymeric micelles described for the delivery of CUR were based on the biodegradable and synthetic copolymer monomethoxy poly(ethylene glycol)-poly(ε-caprolactone) (MPEG-PCL) (Lv et al., 2014; Wang et al., 2013). To the best of our knowledge, a similar approach for CLX (anti-angiogenic effect of CLX loaded polymeric micelles) has not been previously reported in literature. As for CLX, it should be evidenced that in literature, there are conflicting reports concerning the CLX angiogenic activity. While there are several papers showing that this drug is characterized by anti-angiogenic activity (Gately and Li, 2004; Klenke et al., 2006), some published works suggest that CLX might even possess pro-angiogenic activity both when used in free form and encapsulated in PLGA based nanoparticles in normally perfused and ischemic organs (Margulis et al., 2015).

The aim of the present study was to use micelles based on biodegradable and amphiphilic polymers to deliver CUR or CLX, to investigate their role in the control of angiogenesis.

The nanosystem selected by us is based on polymeric micelles composed by inulin as the hydrophilic moiety (Licciardi et al., 2014; Mandracchia et al., 2011; Pitarresi et al., 2008, 2009; Tripodo et al., 2009), and vitamin E as the hydrophobic moiety, giving rise to inulin (INU) vitamin E (VITE) amphiphilic polymer (INVITE), which self-assembles into nanosized micelle able to incorporate highly hydrophobic drugs by established interactions (Catenacci et al., 2014; Mandracchia et al., 2014; Tripodo et al., 2013).

We selected these substances because both INU and the antioxidant VITE are of natural origin from renewable resources. In addition, the choice was motivated considering i) the increasing interest focused to pharmaceutical applications of the flexible oligosaccharide INU; (Mensink et al., 2015) ii) the involvement of VITE in different antitumor process has been demonstrated (Torricelli et al., 2011).

In this study we have investigated the anti-angiogenic activity of CUR or CLX loaded INVITE micelles using the *in vivo* chick embryo chorioallantoic membrane (CAM) assay and the obtained results are herein reported and discussed.

2. Materials and methods

All reagents were of analytical grade, unless otherwise stated. *N*,*N*-dimethylformamide (DMF), triethylamine (TEA), diciclohexylcarbodiimide (DCC), curcumin, celecoxib, pyrene, D-α-tocopherol succinate, poly(acrylic acid), NaCl, KCl, Na_2HPO_4, KH_2PO_4, DMSO-d_6, methanol, dichloromethane (DCM), Tween 20, were purchased from Sigma-Aldrich (Milano, Italy). Inulin from dahlia tubers (INU, approx. 5000 Da), *N*-Hydroxysulfosuccinimide sodium salt (NHSS), Polisorbate 80 were purchased from Fluka (Milano, Italy). DMSO was purchased from Carlo Erba Reagents (Milano, Italy). Dialysis tubes with a MWCO 3.500 Da (Spectra/Por® 6) were purchased from Spectrum Labs.

2.1. Apparatus

FT-IR spectra (KBr pellets) were recorded in the range 4000–400 cm^{-1} using a Perkin–Elmer 1600 IR Fourier Transform Spectrophotometer (Monza, Italy). The resolution was 1 cm^{-1}. UV–Vis analyses were performed by a Spectrometer Lambda 25, Perkin-Elmer, (Monza, Italy).^{1}H-NMR were recorded using a Varian Mercury 300 MHz instrument. Centrifugations were performed with a Beckman Avanti 30 (Milano, Italy) equipped with a temperature control. CLX quantification was performed by a HPLC system Waters (Waters Corp., MA) Model 600 pump equipped with a Waters 2996 photodiode array detector, an UV detector at a wavelength of 250 nm and a C18 Eclypse column (4.6 × 250 mm, 5 mm) from Agilent preceded by a C18 guard column at room temperature.

2.2. Synthesis of INVITE amphiphilic polymer

The synthesis of INVITE amphiphilic polymer has been described previously (Mandracchia et al., 2014). Briefly, 1 g (6.17 mmol) of INU was solubilized in DMF until complete solubilization at 25 °C, then, TEA was added (0.617 mmol). In a separate flask, VITE-succinate (1.23 mmol) was solubilized in DMF under argon, then DCC (2.46 mmol) and NHSS (2.46 mmol) were added and taken under stirring for 3 h at 25 °C. The INU plus TEA solution was added drop to drop to the NHSS activated VITE solution and the reaction carried out under nitrogen for 12 h at 25 °C. Then, the solution was filtered-off to remove the dicyclohexylurea and the resulting clear solution was precipitated in a tenfold excess of acetone with respect to the reaction DMF volume. The same solvent was used to wash the precipitate which was collected at any washing step by centrifugation at 4 °C and 8000 rpm. This procedure resulted effective in INU purification as assessed in other published procedures (Mandracchia et al., 2011; Pitarresi et al., 2009). The INVITE polymer was obtained as dry white-yellow powder which was further purified by a 3 days dialysis process by using a 3500 Da cut-off membrane (Spectra/Por® 6). Then, the water solution was lyophilized and the fluffy solid stored in a desiccator until use. The final product was characterized by ^{1}H-NMR, FTIR, DLS and for its critical aggregation concentration (CAC) to verify the fitting with previous findings (Catenacci et al., 2014; Mandracchia et al., 2014).

ATR-FT-IR (neat): cm^{-1} 3300, 2900, 1740, 1642, 1480, 1400, 1350, 1250, 1125, 1030, 910.

^{1}H-NMR DMSO-d_6: (ppm) 5.17, 4.83, 4.66, 4.07, 3.83, 3.61, 3.52, 2.90, 2.73, 2.53, 1.97, 1.89, 1.74, 1.47, 1.34, 1.22, 1.15, 1.03, 0.93, 0.80.

CAC (as calculated by the pyrene method) (Mandracchia et al., 2014): $1.6 \cdot 10^{-2}$ mM.

Derivatization Degree (DD) as from ^{1}H-NMR (rate integral peak 0.80 ppm (VITE, m, 12H)/integral peak at 3.52–4.07 ppm (fructose ring, m, 7H)): 19.3% mol/mol.

The hydrodynamic size of the INVITE micelles and the polydispersity index, were measured by using the Zetasizer Nano ZS instrument.

In particular, INVITE aqueous solutions at concentrations above CAC were prepared and left to equilibrate overnight at 25 °C under gently stirring. After this time, solutions were filtered by 0.45 μm filter and analyzed by Zetasizer Nano ZS instrument. The measurements were performed in triplicate.

Moreover, INVITE aqueous solutions were used to define the *Z*-potential of the micelles at 1 mg/mL supplemented with KCl 1 mM.