International Journal of Pharmaceutics 441 (2013) 654–664

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical nanotechnology

Glycyrrhetinic acid-graft-hyaluronic acid conjugate as a carrier for synergistic targeted delivery of antitumor drugs

Li Zhang[a,c,1], Jing Yao[a,b,1], Jianping Zhou[a,b,*], Tao Wang[d], Qiang Zhang[e]

[a] Department of Pharmaceutics, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China
[b] State Key Laboratory of Natural Medicines, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China
[c] Department of Laboratory Medicine, Jianye Center for Disease Control and Prevention, 18 Yueanjie, Nanjing 210019, China
[d] New Drug Screening Center, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China
[e] Pharmaceutical Sciences Department, Peking University, 38 Xueyuan Road, Haidian District, Beijing 100191, China

ARTICLE INFO

Article history:
Received 12 July 2012
Received in revised form
24 September 2012
Accepted 19 October 2012
Available online 29 October 2012

Keywords:
Conjugates
Hyaluronic acid
Glycyrrhetinic acid
Synergistic targeted delivery
Liver tumor

ABSTRACT

Glycyrrhetinic acid-graft-hyaluronic acid (HGA) conjugate was synthesized as a carrier for intravenous administration of paclitaxel (PTX), which combined hyaluronic acid (HA) and glycyrrhetinic acid (GA) as the active targeting ligands to liver tumor. In the present study, physicochemical characteristics, cellular uptake efficiency, and *in vivo* fates of HGA conjugates were investigated. HGA nanoparticles could readily load PTX with high efficiency up to 31.16 wt.% and entrapment efficiency to 92.02%. Moreover, PTX-loaded HGA nanoparticles exhibited more significant cytotoxicity to HepG2 cells than B16F10 cells due to simultaneously over-expressing HA and GA receptors. Meanwhile, the cellular uptake of nanoparticles was clearly enhanced in HepG2 and B16F10 cells compared to a normal fibroblast cell (HELF cells). In particular, more HGA nanoparticles were taken up by HepG2 cells than by B16F10 cells, which might be attributed to the affinity of multiple ligands of HA and GA to HepG2 cells. Furthermore, liver and tumor targeting activity of HGA nanoparticles was also confirmed by *in vivo* imaging analysis. The fluorescence signals of DiR-labeled HGA nanoparticles in tumor and liver were 2.88 and 1.83 folds stronger than that of the control, respectively. These results indicate HGA nanoparticles can be a potential drug carrier with "double target sites" for liver cancer therapy.

1. Introduction

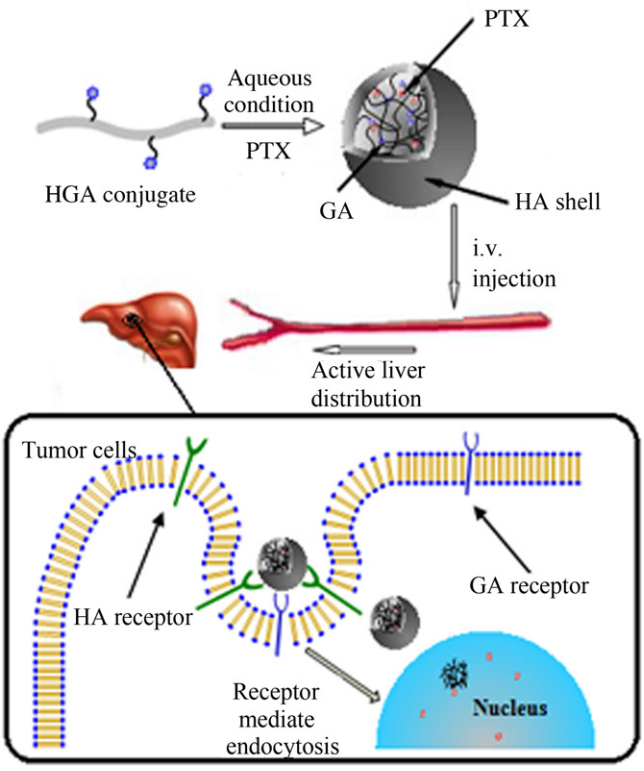
Existing anti-cancer drug deliveries focus on self-assembled polymeric nanoparticles based on implantable biomaterials. Owing to the special core–shell structure of such nanoparticles, they have superior properties *in vitro* and *in vivo*, including high loading capacity of poorly water-soluble drugs, releasing drugs in a sustained manner, thus increasing bioavailability of them (Saravanakumar et al., 2010; Park et al., 2004; Kim et al., 2006; Hou et al., 2011). However, only partial amounts of loaded-drugs reach the target site due to some physiological limitations. For example, generally, the high tumor interstitial fluid pressure contributes to a decreased uptake of drugs in the tumor (Danhier et al., 2010; Heldin et al., 2004). In addition, the extravasation of polymeric nanoparticles will be influenced by tumor types and anatomical sites (Bae, 2009). One approach to overcome these limitations is active targeting strategies such as binding to appropriate receptors highly expressed at the target site.

Hyaluronic acid (HA), a biocompatible polyanionic polysaccharide, has been implicated in several biological functions. It is well known that HA receptors play pivotal biological roles in degradation, endocytosis and signal transduction (Oh et al., 2010). The best-identified HA receptors, such as cluster determinant 44 (CD44), are over-expressed in various kinds of malignant cells (Aruffo et al., 1990; Choi et al., 2010; Lapcik et al., 1998). Although the precise mechanism was unclear, Hua et al. (1993) demonstrated that the unique interaction between HA and CD44 might induce various intracellular signaling pathways to enhance the cellular uptake of HA. Perceptibly, HA can be used as a promising constituent of nanocarrier in anti-cancer drug delivery due to its biodegradability and specific binding to CD44.

Glycyrrhetinic acid (GA) is the metabolite of the natural product glycyrrhizin. It possesses several pharmacological activities, such as anti-inflammatory and immuno-modulating (Asl and Hosseinzadeh, 2008). Furthermore, it has been reported that GA also reverses the multidrug resistance to anti-tumor agents and has potential as a lead compound for the design of less toxic chemosensitizing agents (Nabekura et al., 2008; Lee et al., 2008). Recently, there have been several studies on its strong distribution of liver

* Corresponding author at: Department of Pharmaceutics, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China. Tel.: +86 25 83271102; fax: +86 25 83301606.
E-mail address: zhoujpcpu@163.com (J. Zhou).
[1] Li Zhang and Jing Yao contributed equally to this work.

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijpharm.2012.10.030



Scheme 1. Illustration of the self-assembly, accumulation at liver tumor tissue and intracellular trafficking pathway of HGA nanoparticles. The intracellular trafficking pathway includes steps of receptor-meditated cellular internalization, endo/lysosomal escape, reduction triggered nanoparticles disassembly, and drug release.

cells since a report showed that GA could bind to rat liver parenchymal cell membrane (Negishi et al., 1991; Tian et al., 2010a,b; Mao et al., 2007). It has also been reported that GA could bind to protein kinase C α in the human hepatocellular carcinoma cell line (He et al., 2010). Still, of the liver targeted vectors modified with single functional group – GA, improving the tumor targeting efficiency has been one of the major criteria in designing new vectors for hepatocellular carcinoma chemotherapy.

In this study, an amphiphilic HA derivative conjugated with GA was developed to self-assemble nanoparticles for liver tumor targeting delivery of paclitaxel (PTX) (Scheme 1). PTX, a microtubules stabilizer that causes mitotic arrest, is widely used to treat breast, non-small cell lung, and ovarian cancer (Rowinsky and Donehover, 1995). Moreover, it also showed significant antitumor activity against hepatoma cells in mice (Lin et al., 2000; Fuchs et al., 1999; Liang et al., 2006), additionally in the clinical use (Chen and Xue, 2004). However, it is limited in the practical applications due to its toxicity, short half-life and poor solubility (Geisler et al., 2007; Ganta and Amiji, 2009). In addition, PTX formulations approved by the FDA are non-selectively targeted to tumor cells at present (Rivkin et al., 2010).

Accordingly we have set our aims to give proofs for targeting the liver tumor through combining different promising strategies – the synergistic actions of GA and HA. Therefore, HGA nanoparticles are expected as a novel drug delivery system with "double target sites". We synthesized HGA conjugates with different degree of substitution (DS) of GA and characterized their physicochemical properties. Cytotoxicity of PTX-loaded HGA nanoparticles and the cellular uptake behavior of nanoparticles in various cell lines were also investigated. The targeting distribution of nanoparticles labeled with DiR (a fluorescence dye) to the liver and tumor was evaluated.

2. Materials and methods

2.1. Materials

Hyaluronic acid (10 kDa) was obtained from Shandong Freda Biochem Co. Ltd. (Shandong, China). Paclitaxel (PTX) and glycyrrhetinic acid (GA) were purchased from Chongqing Melian Pharmaceuticals Co. Ltd. (Chongqing, China) and Nanjing Zelang Medicine Technology Co. Ltd. (Jiangsu, China), respectively.

Anhydrous dimethylformamide (DMF), anhydrous formamide, anhydrous dichloromethane (DCM) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were from Shanghai Lingfeng Chemical Reagent Co. Ltd. (Shanghai, China) and Sigma Chemical Co. (St. Louis, MO), respectively. N-Hydroxysuccinimide (NHS), N,N-dicyclohexyl carbodiimide (DCC) and pyrene were from Sinopharm Chemical Reagent Co. Ltd. (Najing, China). All other chemicals were of analytical grade and were used without further purification.

2.2. Synthesis of HGA conjugates

The HGA conjugate was synthesized by coupling HA with aminated GA (Fig. 1). Firstly, GA (3 mmol) in 50 mL DCM was reacted with NHS (3.6 mmol), and then DCC (3.6 mmol) was added to the solution at ambient temperature. After reaction overnight, the precipitant was filtrated off and washed several times with DCM to obtain the succinimido GA. To an ice-cold solution of ethylenediamine (4 mmol) in dichloromethane (DCM) (6 mL), solution of the succinimido GA (1.3 mmol) was added dropwise. Finally, pure GA-NH_2 was obtained by silica gel column chromatography.

Secondly, HA (0.1 g) was dissolved in formamide (10 mL) by gentle heating. Different amounts of EDC and NHS were mixed with HA solutions at room temperature, followed by the addition of different amounts of GA-NH_2 dissolved in DMF (20 mL). The mixture was precipitated in excess cold acetone after 24 h, and the precipitate was washed two times with acetone to remove excess GA-NH_2. The resulting conjugate was dissolved in water and then dialyzed against deionized water for 48 h using a dialysis membrane (MWCO 3500). The residue was freeze-dried to get the HGA conjugate as white floccule.

2.3. Preparation of PTX-loaded HGA nanoparticles

The PTX-loaded HGA nanoparticles were prepared by a dialysis method. Briefly, the PTX solution in ethanol was added into the HGA solution of 6 mg/mL with stirring, and the solution was ultrasonicated for 30 min in ice-bath by an ultrasonicator (JY92-2D, Ningbo Scentz Biotechnology Co., Ltd., China). The resulting solution was dialyzed against the distilled water overnight, filtering through a 0.8 μm microporous membrane and lyophilization.

2.4. Characterization of drug-free and PTX-loaded nanoparticles

The structure of HGA conjugate was analyzed by ^{1}H NMR (Avace AV-500, Bruker, Germany) and FI-TR (Tensor27, Bruker, Germany). The degree of substitution (DS), defined as the number of GA per HA molecule, was estimated by UV measurements (λ = 250 nm). The critical aggregation concentration (CAC) was determined using a fluorescence spectrophotometer with pyrene as the probe. Pyrene solution in acetone (6×10^{-6} M) was prepared, and the acetone evaporated under a gentlenitrogen (N_2) gas stream. HGA solution in H_2O (10 mL), the concentration of which ranged from 1 to 250 mg/mL, was added to each tube to achieve a final pyrene concentration of 6×10^{-7} M. The fluorescence intensity was measured using a fluorescent spectrometer RF-5301 (Shimadzu Co.,