International Journal of Pharmaceutics 478 (2015) 553–568

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical nanotechnology

Hepatocellular carcinoma dually-targeted nanoparticles for reduction triggered intracellular delivery of doxorubicin

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ARTICLE INFO

Article history:
Received 29 June 2014
Received in revised form 24 September 2014
Accepted 15 October 2014
Available online 22 October 2014

Keywords:
Hepatocellular carcinoma targeting
Dual ligand targeting
Hyaluronic acid
Glycyrrhetinic acid
Redox responsive
Antitumor efficacy

ABSTRACT

Hepatocellular carcinoma (HCC) dual targeted stimuli responsive nanoparticles (NPs) for intracellular delivery of doxorubicin (DOX) were developed based on a reduction cleavable hyaluronic acid–glycyrrhetinic acid conjugate (HA-Cyst-GA). HA-Cyst-GA conjugate readily formed NPs in aqueous milieu and exhibited a high drug loading capacity (33.9%). The NPs redox responsiveness evaluation showed a tendency to lose their structural integrity in response to a reductive stimulus while remaining stable at physiological conditions, and that drug release was dramatically accelerated in presence of an intracellular level of glutathione. Moreover, cellular uptake studies highlighted the affinity of hepatoma cells (HepG2) toward the NPs as compared to breast cancer cells (MDA-MB-231). HA-Cyst-GA DOX-NPs displayed an increased cytotoxic potency over their non-responsive counterparts and free DOX with IC50 of 5.75, 9.33 and 10.23 μg/mL, respectively. CLSM observations showed that HA-Cyst-GA DOX-NPs mediated a faster intracellular release and nuclear delivery of DOX as compared to the insensitive control. In vivo imaging study performed on H22 tumor bearing mice revealed a selective accumulation of DiR labeled NPs in the tumor and liver upon systemic administration. The antitumor efficacy was evaluated in HepG2 tumor xenograft model. Overall HA-Cyst-GA NPs appear as a potential HCC targeted intracellular delivery platform for DOX.

1. Introduction

Liver cancer treatment with conventional chemotherapeutic agents, as effective as it might be, can produce severe systemic side effects (Kim and Nie, 2005). The emergence of nanotechnology based drug delivery platforms could meaningfully contribute to the improvement of anticancer drugs therapeutic outcome, notably through the reduction of non-specific interactions by the introduction of adequate targeting abilities (Milane et al., 2011; Peer et al., 2007). In this regard, amphiphilic nanocarriers that can accommodate hydrophobic pharmaceuticals have received a widespread interest (Rösler et al., 2001). Owing to their hydrophilic shell, these drug carriers could elude clearance by the reticulo-endothelial system (RES), and thus prolong their circulation half-life (Kim and Nie, 2005), which ultimately facilitate their accumulation at the tumor site by passive targeting due to the endothelial permeation and retention (EPR) effect (Matsumura and Maeda, 1986; Torchilin, 2011). Nevertheless, potential efficiency of NPs lacking affinity for the target cells is rather restricted, as intracellular delivery of most chemotherapeutic agents is a prerequisite for these pharmaceuticals to exert their action (Muro, 2012; Torchilin, 2006). Therefore, in order to enhance cellular uptake performance through ligand–receptor mediated endocytosis, surface functionalization of NPs with targeting elements known as active targeting has been actively pursued (Bertrand et al., 2014; Muro, 2012; Torchilin, 2006).

Among a plethora of targeting ligands, Hyaluronic acid (HA), a naturally occurring anionic polysaccharide has been extensively investigated as a drug carrier (Ray et al., 2013). Particular emphasis has been placed on HA's binding affinity to CD44 receptor, which is over-expressed by numerous tumor cells including various carcinomas (Orian-Rousseau, 2010). Accordingly, nanocarriers that

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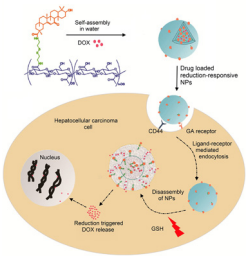
http://dx.doi.org/10.1016/j.ijpharm.2014.10.041
0378-5173/

use HA as a targeting constituent showed an increased cellular uptake through CD44 mediated endocytosis (Cho et al., 2012; Choi et al., 2011, 2010). Furthermore, a non-negligible hepatic accumulation of HA based NPs has been reported following systemic administration (Choi et al., 2010). Probably due to HA's interaction with HARE receptors expressed on liver sinusoidal endothelial cells, and could hypothetically be beneficial for hepatic tissues targeting (Oh et al., 2010 Zhou et al., 2002).

Glycyrrhetinic acid (GA), a triterpenoid saponin is one of the main bioactive compounds extracted from licorice. Early work conducted by Negishi et al. (1991) revealed the existence of GA receptor on rat hepatocytes surface, thus raising the possibility of exploiting GA as a liver targeting moiety. More recent researches identified protein kinase C (PKC) α as GA binding site in human hepatocytes. Their expression is indeed more pronounced on hepatocellular carcinoma cells, suggesting that vehicles modified with GA could discriminate liver tissue and hepatoma tissue (Guo et al., 2013; He et al., 2010; Yang et al., 2003; Ying et al., 2008). Moreover, NPs functionalized with GA have shown promising results regarding liver targeting efficiency compared to their non-functionalized counterparts (Tian et al., 2010, 2012; Zhang et al., 2012).

To achieve an optimal therapeutic action, fast release of the active substance at the desired site is yet another hurdle to overcome. The undeniable benefit of active targeting strategy might, however, be limited by the slow release rate of conventional drug loaded carriers in the target cells (Cheng et al., 2011). Therefore, stimuli sensitive nanocarriers equipped with triggered drug release components have been engineered, such vehicles possess a sophisticated architecture that evolves in response to an biological stimulus, leading to a burst release of their cargo at the target site (Fleige et al., 2012). One elegant approach consisting of exploiting redox potential contrast between extra and intracellular environments. In this regard, drug carriers comprising disulfide bonds within their constituting units have been designed (Meng et al., 2009). These reducible linkages exhibit good stability in the mildly oxidative extracellular milieu, yet undergo a rapid cleavage in the highly reductive intracellular compartments by thiol–sulfide exchange reactions essentially with glutathione (GSH) (Gilbert, 1995). Such dissociation causes an abrupt loss of the nanocarriers structural integrity, thereby initiating a prompt release of their payload (Li et al., 2012; Sun et al., 2009). Moreover, GSH is abundant within cells with concentrations ranging from 0.5 to 10 mM compared to blood plasma and extracellular matrix levels that ranges from 2 to 20 μM (Wu et al., 2004). This difference is even more pronounced in malignant states. Indeed, GSH levels are at least 4-fold higher in hypoxic tumor tissues over normal tissues (Kuppusamy et al., 2002), rendering reduction-sensitive nanocarriers even more suitable for intracellular delivery of pharmaceutical agents to tumors.

In this contribution, we report the development of multi-functionalized NPs for liver cancer targeted reduction triggered intracellular delivery of the chemotherapeutic agent DOX (Scheme 1). The NPs were fabricated by self-assembly of amphiphilic reducibly degradable GA-modified HA polymer. Reduction responsiveness of the NPs was evaluated in vitro by monitoring the disassembly and drug release behavior in response to different concentrations of GSH. In order to evaluate the additional benefit of reduction-sensitive NPs over their hydrolytically degradable analogues, in vitro cytotoxicity and intracellular drug release pattern were investigated in hepatocellular carcinoma HepG2 cells. Cellular uptake via ligand–receptor mediated endocytosis involving either GA or HA ligand was evaluated in HepG2 and MDA-MB-231 cell lines. In vivo distribution of DiR-labeled NPs was monitored by a non-invasive near-infrared (NIR) imaging technique using H-22 tumor bearing mice. Finally, in vivo antitumor activity of DOX loaded NPs was evaluated in HepG2 tumor xenograft mouse model.



Scheme 1. Illustration of self assembly, drug loading, CD44/GA receptors mediated endocytosis and GSH triggered disassembly of HCC dually targeted reduction responsive HA-Cyst-GA nanoparticles leading to intracellular burst release of DOX.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (MW 10 kDa) was purchased from Freda Biochem Co., Ltd. (Shandong, China). Glycyrrhetinic acid (purity ≥98%) was purchased from Nanjing Zelang medical technology Co., Ltd. (Nanjing, China). *N*,*N*′-dicyclohexyl carbodiimide (DCC), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), *N*-hydroxysuccinimide (NHS) and pyrene were obtained from Aladdin Reagent Co., Ltd. (Shanghai, China). Cystamine dihydrochloride, L-glutathione reduced, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and coumarin-6 were purchased from Sigma–Aldrich (St. Louis, Missouri, United States). Doxorubicin hydrochloride was obained from Zhejiang Hisun pharmaceutical Co., Ltd. (Taizhou, China). DiR iodide was purchased from Beijing Fanbo biochemicals Co., Ltd. (Beijing, China). Hoechst 33342 was purchased from Beyotime Institute of Biotechnology (Haimen, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from sunshine biotechnology (Nanjing) Co., Ltd. (Nanjing, China). All other chemicals were of analytical grade.

MDA-MB-231 Human breast carcinoma and HepG2 hepatocellular carcinoma cell lines were obtained from culture collection of the Chinese Academy of Science (Shanghai, China). MDA-MB-231 and HepG2 cells were cultured in L15 (Leibovitz) and RPMI-1640 medium (Hyclone Laboratories, Thermo Scientific), respectively. Both media were supplemented with 2.05 mM L-glutamine, 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 ug/mL streptomycin). The cells were maintained at 37 °C in a humidified atmosphere. HepG2 cells were cultured in the same conditions with 5% CO_2.

2.2. Synthesis of hyaluronic acid–cystamine–glycyrrhetinic acid (HA-Cyst-GA) and hyaluronic acid–ethylenediamine–glycyrrhetinic acid (HA-Etda-GA) conjugates

2.2.1. Neutralization of cystamine dihydrochloride

Cystamine free base was prepared according to the literature (Alferiev et al., 2005). Briefly, cystamine dihydrochloride (25 g, 0.11 mol) was dissolved in distilled water (32.25 mL). Tetahydrofuran (THF) (50 mL) and diethyl-ether (Et_2O) (125 mL) were subsequently added. The mixture was cooled in an ice bath and a 10 N NaOH solution (166.6 mL) was then slowly added dropwise over 1 h. The organic layer was separated and the aqueous layer was further extracted with a mixture of THF (37.5 mL) and Et_2O (105 mL). The combined organic layers were dried over NaOH (10 g) at 4 °C for 2 h. The desiccant was discarded by filtration and additionally washed with a mixture of THF (20 mL) and Et_2O