International Journal of Pharmaceutics 512 (2016) 39–48

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Integrating a novel SN38 prodrug into the PEGylated liposomal system as a robust platform for efficient cancer therapy in solid tumors

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ARTICLE INFO

Article history:
Received 10 June 2016
Received in revised form 26 July 2016
Accepted 17 August 2016
Available online 17 August 2016

Keywords:
Prodrug
Liposome
Supramolecular nanoassembly
Drug delivery
Cancer therapy

ABSTRACT

Liposomal nanoassemblies have been used extensively as carriers for the delivery of both lipophilic and hydrophilic drugs. They represent a mature, versatile technology with considerable potential for improving the pharmacokinetics of drugs. However, the formulation of many chemotherapeutics into liposome systems has posed a significant challenge due to their incompatible physicochemical properties, as was the case with camptothecin-based chemotherapeutics. Here, we present a rational paradigm of potent chemotherapeutics that were reconstructed and subsequently integrated into liposomal nanoassemblies. Using SN38 (7-ethyl-10-hydroxy camptothecin) as a model drug, a lipophilic prodrug **1** (designated as LA-SN38) was constructed by tethering the linoleic acid (LA) moiety via esterification, which was further facilitated to form liposomal nanoparticles (LipoNP) through supramolecular nanoassembly. The resulting **1**-loaded LipoNP exhibited sustained drug release kinetics and decreased cellular uptake by macrophage cells. Uptake by tumor cells was enhanced relative to our previous supramolecular nanoparticles (SNP **1**), which were derived from the self-assembling prodrug **1**. Notably, LipoNP outperformed SNP **1** in terms of pharmacokinetics and *in vivo* therapeutic efficacy in both human BEL-7402 hepatocellular carcinoma (HCC) and HCT-116 colorectal cancer-derived xenograft mouse models. These results were likely due to the improved systemic circulation and preferential accumulation of nanodrugs in tumors. Hence, our results suggest that the combination of liposomal delivery platforms with rational prodrug engineering may emerge as a promising approach for the effective and safe delivery of anticancer chemotherapeutics.

1. Introduction

SN38 (7-ethyl-10-hydroxy camptothecin) is a chemotherapeutic agent belonging to the camptothecin family of highly potent topoisomerase I inhibitors (Pommier, 2006; Sepehri et al., 2014). Despite its promising anticancer potential, this molecule is extremely insoluble in aqueous solutions and pharmaceutically acceptable solvents (e.g., polysorbate 80 and Cremophor EL), thereby hindering its clinical application. Accordingly, Irinotecan (CPT-11), a water-soluble SN38 prodrug, was developed for the treatment of a variety of cancers (Pizzolato and Saltz, 2003; Venditto and Simanek, 2010). CPT-11 can be converted to the active metabolite SN38 by carboxylesterases in liver and tumor tissues, but the metabolic conversion rate is low (less than 8% of the injected dose of CPT-11) (Slatter et al., 2000). Previous *in vitro* studies showed that SN38 was 100- to 1000-fold more potent than CPT-11. Therefore, the direct administration of SN38 could bypass the inefficient enzymatic activation of CPT-11, which would be greatly beneficial for improving the therapeutic index. To resolve this issue, numerous efforts have been devoted to the development of novel formulations for this potent molecule through covalent modifications (Bala et al., 2013; Sayari et al., 2014). Examples of such modifications include the attachment of SN38 agents to water-soluble motifs, including tumor-targeting iRGD by us (Xie et al., 2016), as well as attachment to hydrophilic polymers, such as poly(ethylene glycol) (PEG)-poly(glutamic acid) (e.g., NK012) (Matsumura, 2011), multiarm PEG (e.g., EZN-2208) (Zhao et al., 2008) and antibodies (Moon et al., 2008; Sharkey et al., 2012).

Alternatively, our lab (Wang et al., 2014b) and others (Alferiev et al., 2015) have recently demonstrated strategies utilizing molecular edits to enhance the lipophilicity of the SN38 molecule, which gave the produced prodrugs the ability to assemble with amphiphilic polymer matrices. More intriguingly, we found that

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http://dx.doi.org/10.1016/j.ijpharm.2016.08.036
0378-5173/

the esterification of 10-hydroxyl on the SN38 agent facilitated the self-assembly of the SN38 prodrugs into supramolecular nanoparticles (SNPs) in aqueous media (Wang et al., 2015). Among a library of SN38 derivatives that tether a structural diversity of lipophilic moieties, we found that those featuring polyunsaturated alkyl chains were more capable of forming SNPs than others independent of excipients. Though our pure prodrug-composed platforms were effective in retarding tumor growth *in vivo*, they suffered from inadequate pharmacokinetics, representing the 'Achilles heel' of this fabrication approach. This unsolved issue prompted us to explore sophisticated strategies that could prolong the systemic circulation of therapeutic SN38 agents in the bloodstream, which may be essential for achieving optimal delivery of nanodrugs into solid tumor lesions (Termsarasab et al., 2014).

Liposomal nanoassemblies represent a mature, versatile technology with considerable potential for entrapment of both lipophilic and hydrophilic drugs (Al-Jamal and Kostarelos, 2011; Allen and Cullis, 2013; Seeta Rama Raju et al., 2015). Because of their robust biocompatibility and versatile drug loading capacity, there are several human therapeutic applications, such as doxorubicin (Hadjidemetriou et al., 2016), cisplatin (Sengupta et al., 2012; Zhou et al., 2015) and siRNA (Buyens et al., 2012; Wang et al., 2014a). Encapsulation of therapeutics within liposomes often confers the drugs with excellent *in vivo* properties, including improved pharmacokinetics and pharmacodynamics relative to the corresponding free drugs. In addition, passive accumulation of drugs in solid tumors via the enhanced permeation and retention (EPR) effect was observed, which could contribute to the markedly reduced drug toxicity and enhanced therapeutic index (Jiang et al., 2014; Maeda et al., 2000).

In this article, we report on the development of a liposome-based delivery system for encapsulation of a lipophilic SN38 prodrug with the aim of improving *in vivo* pharmacokinetics and therapeutic efficacy. Based on our previous design, we utilized the optimal derivative **1** tethering the polyunsaturated fatty acid linoleic acid (LA) to facilitate supramolecular assembly in liposomal vehicles (Fig. 1A). The prodrug **1** was able to form colloidal stable liposomal nanoparticles (designated as LipoNP) that were able to tolerate exceptionally high drug loading. Furthermore, *in vitro* cytotoxicity and *in vivo* antitumor efficacy were evaluated in several cancer cell lines and cancer cell-derived xenograft mouse models, respectively.

2. Materials and methods

2.1. Materials

LA-SN38 prodrug **1** was synthesized according to our previous report. (Wang et al., 2015) 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000] (DSPE-PEG_{2000}), cholesterol (Chol) and egg phosphatidylcholine (Egg-PC) were purchased from Advanced Vehicle Technology Pharmaceutical Ltd. (Shanghai, China). Penicillin, streptomycin, RPMI 1640, fetal bovine serum (FBS), 0.25% (w/v) trypsin, and 0.03% (w/v) EDTA solution were purchased from HyColon. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Shanghai, China). The DiR (D12731) probe was purchased from Life Technologies (Shanghai, China).

2.2. Preparation of SN38 prodrug 1-loaded liposomal nanoparticles (LipoNPs)

LipoNPs were prepared by ethanol dilution as described by Bo Yu et al. (2012). Briefly, the lipid mixture of Egg-PC, cholesterol and DSPE-PEG_{2000} was first prepared in ethanol (900 μL) at a molar ratio of 30:15:1. The prodrug **1** (at a concentration of 10 mg/mL in 100 μL of dimethyl sulfoxide, DMSO) was then added to the above ethanolic solution with the ratio of lipids to prodrugs fixed at 10:1 (w/w). The mixture (1 mL) was then rapidly injected into deionized water (9 mL). Finally, the resulting **1**-formulated LipoNPs were reprecipitated at 100000*g* for 20 min using ultracentrifugation (Berkman, Optima™ L-100 XP Ultracentrifuge) and washed with water three times to remove the organic solvents.

2.3. Characterization of drug-loaded NPs

The particle size and zeta potential (ξ) of LipoNP was measured using a Malvern Zetasizer (Nano-ZS90, Malvern, Worcestershire, UK) at 25 °C. The morphology was observed by transmission electron microscopy (TEM) using TECNAL 10 (Philips) at an acceleration voltage of 80 kV.

2.4. Drug encapsulation efficiency and in vitro drug release kinetics

The drug-loading capacity (LC) and encapsulation efficiency (EE) of prodrug **1** in liposomes were determined by extracting 5 mg of drug-loaded LipoNP with 100 mL acetonitrile for 1 h while stirring. The samples were then filtered and analyzed by HPLC. Drug loading (%) = (weight of drug in LipoNP/weight of LipoNP) × 100. Encapsulation efficiency (%) = (weight of drug in LipoNP/weight of drug fed initially) × 100.

The *in vitro* release kinetics of prodrug **1** from NPs were quantified by dialyzing against phosphate buttered saline (PBS, pH 7.4) containing 0.2% Tween 80. Briefly, NP solutions containing the prodrug **1** (at an SN38 equivalent concentration of 0.1 mg/mL) were end-sealed in dialysis tubes (Spectrum, a molecular weight cutoff of 14 kD) and continuously stirred in an orbital shaking water bath at the speed of 100 rpm. At pre-determined time intervals, the release media were collected and equal volumes of fresh media were supplemented. The total amounts of released SN38 in the samples were determined by HPLC analysis. The HPLC analyses were performed under the follow conditions: The mobile phase was a gradient of water (A) and acetonitrile (B) at a flow rate of 1.0 mL/min. The linear gradient was 40% A:60% B to 0%A:100% B within 15 min; then 0% A:100% B for 10 min. UV detection at a wavelength of 380 nm was performed, and a C18 ODS reverse-phase column (5 μm, 250 mm × 4.6 mm, YMC Co., Ltd., Kyoto, Japan) was utilized. The release kinetics were calculated as a function of the incubation time.

2.5. Cell lines and cell culture

Human colon carcinoma HCT-116 and human HCC BEL-7402 cells were maintained in RPMI 1640. Human colon carcinoma HT-29 and Huh-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM). All of the media were supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL), and the cells were maintained in a humid atmosphere at 37 °C with 5% CO_2.

2.6. Intracellular uptake

HCT-116 human colorectal cancer cells and RAW264.7 murine macrophage cells were cultured in RPMI 1640 supplemented with 10% (v/v) FBS and 20% (v/v) FBS, respectively, along with 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 °C and 5% CO_2.

To investigate the cellular uptake of LipoNP, HCT-116 and RAW264.7 cells were seeded in six-well plates at a density of 6×10^5 cells/well in 2 mL of culture medium. After 24 h, the medium was replaced with fresh medium containing SNP **1** and LipoNP (at an SN38 equivalent concentration of 100 μg/mL) and incubated for 1, 2, 4, 6, and 12 h at 37 °C. At the end time of the incubation, cells were washed with PBS three times to remove excess drugs and then harvested by centrifugation at 3000 rpm for 5 min. RIPA buffer (pH