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Paclitaxel isomerisation in polymeric micelles based on hydrophobized hyaluronic acid

Daniela Šmejkalová [a,*], Kristina Nešporová [a], Martina Hermannová [a], Gloria Huerta-Angeles [a], Dagmar Čožíková [a], Lucie Vištejnová [a], Barbora Šafránková [a], Jaroslav Novotný [a], Jiří Kučerík [b], Vladimír Velebný [a]

[a] Contipro Pharma, Dolní Dobrouč 401, 561 02 Dolní Dobrouč, Czech Republic
[b] University of Koblenz-Landau, Campus Landau, Institute for Environmental Sciences, Fortstraße 7, 768 29, Landau, Germany

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ABSTRACT

Physical and chemical structure of paclitaxel (PTX) was studied after its incorporation into polymeric micelles made of hyaluronic acid (HA) (M_w = 15 kDa) grafted with C6 or C18:1 acyl chains. PTX was physically incorporated into the micellar core by solvent evaporation technique. Maximum loading capacity for HAC6 and HAC18:1 was determined to be 2 and 14 wt.%, respectively. The loading efficiency was higher for HAC18:1 and reached 70%. Independently of the derivative, loaded HA micelles had spherical size of approximately 60–80 nm and demonstrated slow and sustained release of PTX *in vitro*. PTX largely changed its form from crystalline to amorphous after its incorporation into the micelle's interior. This transformation increased PTX sensitivity towards stressing conditions, mainly to UV light exposure, during which the structure of amorphous PTX isomerized and formed C3—C11 bond within its structure. *In vitro* cytotoxicity assay revealed that polymeric micelles loaded with PTX isomer had higher cytotoxic effect to normal human dermal fibroblasts (NHDF) and human colon carcinoma cells (HCT-116) than the same micelles loaded with non-isomerized PTX. Further observation indicated that PTX isomer influenced in different ways cell morphology and markers of cell cycle. Taken together, PTX isomer loaded in nanocarrier systems may have improved anticancer activity *in vivo* than pure PTX.

1. Introduction

Paclitaxel (PTX) is one of the most successful cancer therapeutic agent, which is currently used for chemotherapy of patients with lung, ovarian, breast, head and neck cancer and advanced forms of Kaposi's sarcoma (Lee et al., 2012). The major disadvantage, limiting PTX application in cancer chemotherapy, is its poor aqueous solubility (approximately 1 μg/mL) and low therapeutic index (Kim et al., 2006). Currently, in common commercial composition, PTX solubility is enhanced by using a Cremophor EL: ethanol mixture (50:50 v/v), which is diluted with saline or dextrose solution prior administration. However, there are some problems employing this formulation. The major problem is associated with the presence of Cremophor EL, which serves as surfactant and stabilizer, but at the same time causes a number of undesirable side effects including hypersensitivity reactions, nephrotoxicity and neurotoxicity (Shuai et al., 2004). Cremophor EL was also noted to influence endothelial and vesicular muscle function and cause vasodilation, labored breathing, lethargy and hypotension (Singla et al., 2002). In addition, Cremophor formulation was reported to be incompatible with components of infusion sets. Both ethanol and Cremophor were found to leach diethylhexylphtalate (DHEP) from polyvinyl chloride infusion bags and administration sets. The amount of DHEP leakage depends on the concentration of PTX vehicle, length of contact time with container and the type of administration set (Singla et al., 2002).

For the above-mentioned reasons, innovative nanocarrier systems have been often investigated as alternative vehicles of chemotherapeutics. These nanocarrier systems are water soluble and they are able to dissolve hydrophobic drugs including chemotherapeutics within their hydrophobic domains. Taking into account only vehicles with non-covalent PTX binding, PTX has been so far incorporated in polymeric micelles, liposomes, microspheres and nanoparticles (Wei et al., 2009; Zhou et al., 2013). Due to the fact that most of nanocarrier systems are reported to increase PTX aqueous solubility, prolong blood circulation time of drug and reduce nonspecific uptake by reticuloendothelial system (Vlerken et al., 2007), they are very

* Corresponding author. Tel.: +42 0465519569; fax: +42 0465543793.
E-mail address: daniela.smejkalova@contipro.com (D. Šmejkalová).

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promising candidates for drug delivery systems. However, what is not usually concerned is the stability of PTX after incorporation into the carries.

PTX is known to be relatively unstable molecule in solution (Chen et al., 1994; MacEachern-Keith et al., 1997). In cell culture media, PTX was found to be susceptible to hydrolysis and epimerization. The concentration of original PTX in these media was decreasing with time, and PTX was primarily converted to 7-epipaclitaxel (Ringel and Horwitz, 1987), a thermodynamically more stable isomer. Similar isomerization was observed upon PTX heating in the dry state and in organic solvents (MacEachern-Keith et al., 1997). In another study (Volk et al., 1997) focused on profiling PTX degradants, 7-epipaclitaxel together with baccatin III, PTX side chain methyl ester and 10-deacetylpaclitaxel were formed as degradation products when PTX was exposed to basic conditions. Stressing PTX with acid conditions resulted in the formation of 10-deacetylpaclitaxel and oxene ring opened product. Exposure to high intensity light produced a number of degradation products, mainly pentacyclic PTX isomer with a bond between C3 and C11 (Chen et al., 1994; Volk et al., 1997).

Despite the reported instability of PTX in solutions, there is only limited information on the chemical stability of PTX in drug delivery vehicles. In general, the drug may even change its phase (from crystalline to amorphous) when it is incorporated into drug delivery vehicles (Hu et al., 2007; Nepal et al., 2010) and in this way may become more susceptible towards stressing conditions. It is the aim of this work to compare the phase and structure of PTX before and after physical incorporation in polymeric micelles based on hydrophobized (acylated) hyaluronic acid (HA) and to see whether incorporated PTX is more susceptible to common stress conditions used during polymeric micelle preparation and/or lab sterilization practices. A basic analytical characterization of PTXHA systems, including loading capacity, morphology and drug release study will be also provided.

2. Materials and methods

2.1. Materials

Hyaluronic acid (M_w = 15 kDa) was provided by Contipro Pharma, Dolní Dobrouč, Czech Republic. 4-dimethylaminopyridine (DMAP) was obtained from Merck. Tetrahydrofurane (THF), isopropanol (IPA), triethylamine (TEA), *cis*-oleic acid (OA) and sodium chloride were obtained from Lach-ner (Czech Republic). Hexanoic acid and 2,4,6-trichlorobenzoyl chloride (TCBC) are commercially available products from Sigma–Aldrich. Paclitaxel was obtained from LC Laboratories (New Boston, MA). D_2O and deuterated acetonitrile (ACN_d6) were obtained from Sigma–Aldrich (Czech Republic). Protease and phosphatase inhibitor cocktail tablets were obtained from Roche Diagnostic (Mannheim, Germany), RC DC protein assay kit from Rio-Rad (Hercules, CA), Laemmli buffer from Bio-Rad, rabbit polyclonal (Phospho-cdc2 (Tyr15), Cyclin B1, monoclonal (Phosphor–Histone H3 (Ser10)), p21 Waf1/Cip1 (12D1)) and HRP-linked anti-rabbit IgG antibodies from Cell Signaling (Danvers, MA). SuperSignal West Pico Chemiluminescent Substrate was obtained from Pierce (Rockford, USA) and CP-B X-ray films from Agfa (Brno, Czech Republic).

2.2. Synthesis and characterization of acylated hyaluronan

2.2.1. Oleyl hyaluronan (HAC18:1)

Hyaluronic acid (0.5 g, 1.25 mmol) was dissolved overnight in 10 mL of distilled water. To that solution, 5 mL of tetrahydorurane were slowly added, followed by triethylamine (0.5 mL, 3.75 mmol) and DMAP (0.008 g, 0.0625 mmol). In a second reaction flask, oleic acid (0.38 g, 1.25 mmol) was activated with 2,4,6 trichlorobenzoyl chloride (0.30 g, 1.25 mmol) in the presence of TEA (0.5 mL, 3.75 mmol) in tetrahydrofurane (5 mL). The formation of the aromatic carboxylic anhydride was carried out for 30 min at room temperature (25 °C). Then, the solution containing the mixed aromatic anhydride was added to the solution containing the polysaccharide. The mixture was allowed to react for 2 h at room temperature under vigorous stirring to ensure a good homogenization of the reaction components. The crude product was isolated by precipitation with the addition of 10 mL of super-saturated solution of sodium chloride. After that, the product was washed with an excess of anhydrous isopropanol (50 mL). The product was washed again with aqueous isopropanol solution (85% v/v, 4 × 50 mL). Finally, the precipitate was washed two more times with isopropanol. The white precipitate was decanted and dried in an oven at 40 °C for at least 24 h. The degree of substitution (DS) (in mol%, *i.e.*, moles of fatty acid to moles of HA dimer) was obtained by normalizing the integral of the anomeric proton HA signals from 4.6 ppm to 4.3 ppm to 67 and reading the integral value at δ = 0.9 ppm corresponding to the terminal methyl group of fatty acid. The DS of HAC18:1 was determined to be 10%. ^{1}H NMR (D_2O): acyl signals: δ 0.9 ppm (t, 3H, —CH_2—CH_3), δ 1.2–1.4 and 2.0 ppm (m, 24H, (—CH_2—)$_{12}$), δ 1.6 ppm (m, 2H, —CH_2—CH_2—CO—), δ 2.4 ppm (t, 2H, —CH_2—CO—), δ 5.5 ppm (m, 2H, CH═CH); HA signals: δ 2.0 ppm (—$CONHCH_3$), δ 3.4–3.9 ppm (m, 10H, skeletal, CH), δ 4.4–4.6 ppm (m, 2H, anomeric CH).

2.2.2. Caproyl hyaluronan (HAC6)

Synthetic procedure was similar to HAC18:1, except for the fact that instead of oleic acid, caproyl acid (1.2 mL, 3.75 mmol) was activated with 2,4,6 trichlorobenzoyl chloride (0.90 g, 3.73 mmol) in the presence of TEA (0.5 mL, 7.5 mmol) in tetrahydrofurane (5 mL). The DS was determined from ^{1}H NMR spectra to be 60%. ^{1}H NMR (D_2O): acyl signals: δ 2.4 ppm (m, 2H, α CH_2), δ 1.6 ppm (m, 2H, β CH_2), δ 1.3 ppm (m, 4H, γ, δ CH_2), δ 0.8 (m, 3H, CH_3); HA signals: δ 2.0 ppm (—$CONHCH_3$), δ 3.4–3.9 ppm (m, 10H, skeletal, CH), δ 4.4–4.6 ppm (m, 2H, anomeric CH).

2.3. Preparation of PTX-loaded polymeric micelles

Paclitaxel loaded micelles were prepared by solvent evaporation method. 3.5–20 mg of paclitaxel (Table 1) were dissolved in isopropanol and slowly added to 10 mL of 1% HAC6 or HAC18:1 aqueous solution. The organic solvent was removed by rotary evaporation till a thin film was achieved. The dried film was further hydrated with 10 mL of water. Non-incorporated paclitaxel was removed by filtration (1 μm glass fiber syringe filter) and the resulting filtrate containing polymeric micelles was freeze-dried.

Table 1
Loading efficiency, loading capacity and particle size of paclitaxel (PTX) loaded polymeric micelles HAC6 and HAC18:1. (DS = degree of substitution)

Sample	PTX/HA (mg/mg)	Loading capacity (wt.%)	Loading efficiency (wt.%)	Particle size (nm)[a]
HAC6, DS = 60%	5/100	2.2 ± 0.3	44 ± 6	57 ± 8
HAC18:1, DS = 10%	3.5/100	2.0 ± 0.4	70 ± 11	61 ± 9
HAC18:1, DS = 10%	20/100	14.0 ± 2.0	70 ± 10	66 ± 8

[a] Determined by Cryo-SEM.