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Vincristine-loaded liposomes prepared by ion-paring techniques: Effect of lipid, pH and antioxidant on chemical stability

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Vincristine (PubChem CID: 5978)
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Methanol (PubChem CID: 887)
Chloroform (PubChem CID: 6212)
Tocopherol (PubChem CID: 14,985)
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ABSTRACT

In the present study, vincristine (VCR)-loaded liposomes were designed by ion-pairing techniques and the model could be applied to investigate the effect of lipids on the degradation of vinca alkaloids, and how to weaken their influence by adjusting pH and adding antioxidants. It was found that there was a positive correlation between degree of degradation and the unsaturation extent of the phospholipids. In the phospholipid with the lowest oxidation index, only 6% of VCR was degraded in 6 days at 37 °C, whereas for the phospholipids with highest oxidation index, the degradation reached above 95% over the same time. At pH 6.8 and 7.4, the degradation rate of VCR in the lipid membrane was significantly faster than that in aqueous solution, instead, at pH 5.0. After the addition of butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tocopherol, ascorbate and tocopherol with ascorbate, the residual content of VCR after 6 days was 79.9%, 78.1%, 7.1%, 89.6% and 94.6% respectively. It was speculated that VCR could be oxidized by hydrated peroxyl radicals, which formed from lipid peroxidation as well as nucleophilic substitution with peroxyl radicals in the dry state. Also, the antioxidants were shown to have different eliminating capacity on the peroxyl radicals whether hydrated or not, and the phenoxyl radicals generated from fat-soluble antioxidants may be potentially destabilizing to VCR. Therefore, for these two crucial reasons, the degradation of VCR was quite different when used with a combination of water and fat-soluble antioxidants, and thus provides the best protection for VCR.

1. Introduction

The alkaloids vincristine (VCR) and vinblastine (VLB) were originally isolated from the periwinkle plant *Catharanthus roseus* (El-Sayed and Cordell, 1981). In cell biology, they are able to bind to tubulin dimers, which prevents normal microtubule formation and arrests mitosis in the metaphase, and therefore they have found wide use for the treatment of cancers (Himes, 1991). However, they have low specificity for cancer cells alone, and undergo indiscriminate attacks on all rapidly dividing cells, leading to neurotoxicity, bone marrow suppression and gastrointestinal disorders (Checchi et al., 2003). Based on this, efforts have been made to synthesis derivatives that still maintain activity, but reduce non-specific toxicity, such as vinorelbine (VRB) (Damen et al., 2009). Thus far, drug delivery systems have been used as an effective approach to overcome the problem of non-specific toxicity. In 2012, Marqibo®, a formulation of VCR which loads the drug in sphingomyelin and cholesterol liposomes, was approved by the FDA for the treatment of philadelphia chromosome-negative acute lymphoblastic leukemia. The product could successfully prolong the circulation time and increase the dosage without increasing toxicity (Silverman and Deitcher, 2013).

Vinca alkaloids are unstable *in vivo* and *in vitro*, with typical degradation pathways of oxidation and hydrolysis. It has been extensively reported that vinca alkaloids can be degraded by both endogenous and exogenous oxidizing substances. Schlaifer et al. (1996) have reported that the degradation of Vinca alkaloids could be catalyzed by myeloperoxidase in the presence of H_2O_2. Following on from this, Ünsal Özgen et al. (Ozgen et al., 2000; Özgen et al., 2003) demonstrated that myeloperoxidase could catalyze the formation of HOCl from chloride ions and H_2O_2, and that HOCl mediated the final step in degradation. The strong oxidative effect of sodium hypochlorite on vinca alkaloids was also confirmed by Negreira et al. (Negreira et al., 2016).

Abbreviations: VCR, vincristine; VLB, vinblastine; VRB, vinorelbine; CHEMS, cholesteryl hemisuccinate; VIP, VCR-CHEMS ion-pair complex; VIPL, VCR-CHEMS ion-pair complex liposomes; VCRS, vincristine aqueous solution; HSPC, hydrogenated soybean phosphatide; DPPC, dipalmitoyl phosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TBA, thiobarbituric acid; MDA, malondialdehyde

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Additionally, oxidation of vinca alkaloids catalyzed by CYP3A enzymes, peroxidase and ceruloplasmin has been extensively investigated (Ahn et al., 1997; Dennison et al., 2006).

In an aqueous environment, the main route of degradation is hydrolysis. The most important hydrolysate has been identified as the deacetyl vinca alkaloids of vindoline, which is also the major metabolite in the blood (Black et al., 1988; Damen et al., 2009; Sethi and Thimmaiah, 1985; Thimmaiah and Sethi, 1985). After degradation, the anti-tumor activity of vinca alkaloids may decrease, and it is unknown whether the degradation products will further increase toxicity. Therefore, it is vital to identify a formulation that is capable of protecting vinca alkaloids from degradation. Liposomal delivery systems are often used to improve drug stability, however in this case, the protective effect for vinca alkaloids was not as good as expected, as the formulation either had to be freeze-dried, or used immediately after preparation, similar to Marqibo®. The factors that affect the stability of vinca alkaloids loaded within liposomes are seldom investigated, particularly, the degradation of vinca alkaloids caused by lipids is under-investigated.

In this study, the ion-pair was successfully prepared with VCR and cholesteryl hemisuccinate (CHEMS). Due to the strong interaction between the phospholipids and the CHEMS, VCR could be loaded into the lipid bilayers. Theoretically, the liposome structure remains similar to blank liposomes prepared by phospholipids and CHEMS, with the exception that a portion of the CHEMS is replaced by the ion-pairs. In addition to expectations of hydrolysis after exposure to aqueous medium, the degradations of VCR caused by lipids have been investigated in detail. Detailed studies were performed in order to determine the degradation of VCR loaded in liposomes in different pH environments, as well as the effect of a various phospholipids on the degradation of VCR under both hydration and dry states. By comparing the oxidation degree of different phospholipids over time with the timing of VCR degradation, as well as changes in the content of impurities in the hydration state with the dry state, possible degradation mechanisms could be suggested. Finally, studies on the influence of antioxidants on the degradation of VCR were carried out. In this paper, the role of antioxidants in VCR degradation is discussed and recommendations are made on the use of antioxidants when the VCR were encapsulated in liposomes.

2. Materials and methods

2.1. Materials

Vincristine sulfate was purchased from Guangzhou Han Fang Modern Pharmaceutical Co., Ltd. (Guangzhou, China). Cholesteryl hemisuccinate (CHEMS) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Phospholipids PC-98T (PC (phosphatidylcholine), 98.6%; PE (phosphatidylethanolamine), < 0.1%; OI (oxidation index), 0.51) was provided by Shanghai A.V.T. Pharmaceutical L.T.D. (Shanghai, China). Lipoid E80 (PC, 82.5%; PE, 8.2%; OI, 0.20), Lipoid E80S (PC, 70.0%; PE, 14.9%; OI, 0.15), Lipoid S75 (PC, 69.2%; PE, 9.5%; OI, 0.30), Lipoid S100 (PC, 97.1%; PE, < 0.1%; OI, 0.36), hydrogenated soybean phosphatide (HSPC) and dipalmitoyl phosphatidylcholine (DPPC) were all purchased from Lipoid GmbH (Ludwigshafen, Germany). Vitamin C, tocopherol, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), thiobarbituric acid and tetraethoxypropane were offered by Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid and trichloroacetic acid were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other reagents used were of chromatographic or analytical grade, purchased from Tianjin Concord Technology Co., Ltd. (Tianjin, China).

2.2. Methods of VCR quantification

The quantification was performed on a Hitachi-Chromaster (Tokyo, Japan) equipped with a 5410 UV detector, a 5310 Column Oven, a 5210 Autosampler and a 5110 Pump. The desired component was separated by a HiQ Sil C18 reverse phase column (250 mm × 4.6 mm, 5 μm; Bohus, Sweden). Every 20 μL sample was eluted for 18 min by the mobile phase consisting of methanol, ultrapure water and diethylamine (70/30/0.5, V/V/V, pH 7.0, adjusted by phosphoric acid) at a flow rate of 1.0 mL/min at 35 °C and monitored at 298 nm (Zhang et al., 2013).

2.3. Preparation and characterization of VIP

Vincristine sulfate was dissolved in double-distilled water to a concentration of 100 mg/mL, to which a small volume of $NaHCO_3$ solution was added (1:4, $M_{VCR}:M_{NaHCO3}$). After vortexing vigorously for 5 min, VCR was extracted with 2.5 mL of chloroform three times, followed by solvent evaporation under reduced pressure at 30 °C to collect the product. VCR and CHEMS at a molar ratio of 1: 2 were dissolved in chloroform to a VCR concentration of 10 mg/mL. VCR-CHEMS ion-pair complex (VIP) was obtained by stirring in an ice bath (0–6 °C) for 30 min, followed by removal of chloroform under reduced pressure at 35 °C (Zhang et al., 2013).

The VCR, CHEMS, physical mixture (PhM) and VIP were prepared as KBr disks and the structures were analyzed by an IFS 55 FTIR system (Bruker Optics, Germany).

The VCR, CHEMS and VIP of about 5 mg were dissolved in an appropriate amount of DMSO-d_6 respectively and transferred to the NMR sample tubes. The structures were analyzed by a Bruker AV-600 spectrometer at 600 M (Bruker Optics, Germany).

2.4. Solubility of VCR and VIP in different aqueous medium

An excess of VCR or VIP was dispersed in 3 PBS solutions, PBS-1(0.01 M, pH 7.4), PBS-2(0.01 M, pH 7.4, 0.85% NaCl) and PBS-3(0.01 M, pH 7.4, 4.72% glucose), and then the oversaturated solutions were shaken in a shaking bath (HZQ-C, Dongming Medical Instrument Co., Ltd., Harbin, China) operated at 100 rpm and 37 °C. 72 h later, all samples were centrifuged at 14000 rpm for 10 min. The supernatant was passed through a 0.45 μm microporous filter, then the filtrate was determined by HPLC. All solubility determinations were carried out in triplicate.

2.5. Preparation of VIPL

2.5.1. Ethanol injection method

Briefly, required amounts of phospholipids, CHEMS and VIP (10:2:1, w/w/w) were dissolved in a small amount of ethanol (phospholipid/ethanol, 400:1, w/v). The resulting organic phase was instilled by a syringe into a defined volume of PBS (0.01 M, pH 7.4, ethanol/PBS, 1:10, v/v) under magnetic stirring (700 rpm) at 25 °C/55 °C. After 10 min, the VCR-CHEMS ion-pair complex liposomes (VIPL) was obtained by passing the liposomes 15 times through a liposome extruder with a 50 nm polycarbonate membrane.

2.5.2. Thin-film dispersion method

Briefly, the same lipid mixture as above was dissolved in chloroform, and then the solvent was removed under reduced pressure at 35 °C. Spontaneous liposome formation occurred after the dried film was hydrated with prescribed amounts of PBS. Finally, the liposome suspension was kept stirring for 15 min at 40 °C followed by extrusion as above. This process was for the formulation containing lipoid E80 or E80S only.

2.6. The effect of pH on stability of VCRS and VIPL

2.6.1. The degradation kinetics of VCR in aqueous solution

A series of vincristine aqueous solution (VCRS) containing 40 μg/mL VCR were prepared by adding the VCR stock solution (VCR aqueous

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