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Development and evaluation of transferrin-stabilized paclitaxel nanocrystal formulation

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ABSTRACT

The aim of the present study was to prepare and evaluate a paclitaxel nanocrystal-based formulation stabilized by serum protein transferrin in a non-covalent manner. The pure paclitaxel nanocrystals were first prepared using an antisolvent precipitation method augmented by sonication. The serum protein transferrin was selected for use after evaluating the stabilizing effect of several serum proteins including albumin and immunoglobulin G. The formulation contained approximately 55–60% drug and was stable for at least 3 months at 4 °C. *In vivo* antitumor efficacy studies using mice inoculated with KB cells demonstrate significantly higher tumor inhibition rate of 45.1% for paclitaxel-transferrin formulation compared to 28.8% for paclitaxel nanosuspension treatment alone. Interestingly, the Taxol® formulation showed higher antitumor activity than the paclitaxel-transferrin formulation, achieving a 93.3% tumor inhibition rate 12 days post initial dosing. However, the paclitaxel-transferrin formulation showed a lower level of toxicity, which is indicated by a steady increase in body weight of mice over the treatment period. In comparison, treatment with Taxol® resulted in toxicity issues as body weight decreased. These results suggest the potential benefit of using a serum protein in a non-covalent manner in conjunction with paclitaxel nanocrystals as a promising drug delivery model for anticancer therapy.

1. Introduction

Paclitaxel is a naturally occurring diterpenoid extracted from the bark of the Pacific yew tree (*Taxus brevifolia*) [1]. In clinical trials paclitaxel has demonstrated antitumoral activity through high-affinity binding to microtubules, stabilizing and enhancing tubulin polymerization and suppression of spindle microtubule dynamics [2–4]. These activities effectively inhibit cell mitosis, motility and intracellular transport, which lead to apoptotic cell death. Unfortunately, clinical advances of paclitaxel in its natural form have been limited by its physicochemical property, more specifically its poor aqueous solubility (~0.3 μg/ml) [5]. The lack of a functional group in the structure of the paclitaxel molecule makes chemical modification of the natural molecule to increase solubility difficult [6]. Therefore, the selection of appropriate delivery platforms to deliver paclitaxel is particularly crucial to the clinical advancement of this antitumor compound.

Various paclitaxel delivery systems have been investigated to improve the solubility and pharmacological properties of paclitaxel, including micelles, liposomes, microparticles, nanoparticles and the use of cosolvents and cyclodextrins [7–10]. The most widely known delivery platform is a cosolvent system consisting of a 50:50 mixture of Cremophor EL® (a polyoxyethylated castor oil) and ethanol. The corresponding formulation (Taxol® or generic equivalents) consists of paclitaxel dissolved at a concentration of 6 mg/ml in the aforementioned cosolvent system, and is administered intravenously following dilution with normal saline or 5% dextrose solution [11]. While this approach overcomes the limiting solubility of paclitaxel, the use of Cremophor EL has been associated with serious and dose-limiting toxicities. More specifically, Cremophor EL has been known to leach plasticizers from standard intravenous tubing, releasing di(2-ethylhexyl)phthalate (DEHP) [1]. The infusion of DEHP has been demonstrated to produce a histamine release and result in hypersensitivity reactions in 20–40% of unpremedicated patients in phase I clinical trials [12]. Furthermore, Cremophor EL has also been associated with hyperlipidemia, erythrocyte aggregation, sensory neuropathy and neutropenia [13–15]. Clearly, there is a need to develop alternative delivery systems for paclitaxel that enhance drug solubility while eliminating adverse reactions.

A possible alternative delivery system for paclitaxel is a nanosuspension formulation. A nanosuspension consists of nanosized, crystalline particles that may or may not be stabilized by a suitable stabilizer or multiple stabilizers [16,17]. Nanosuspension formulations of several drugs are already marketed, including Rapamune® (sirolimus), Emend® (aprepitant) and Tricor® (fenofibrate) [18]. There are several advantages to using nanosuspension formulations for

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delivery of anticancer agents such as paclitaxel: (i) nanosized particles can enhance dissolution velocity and saturation solubility of a poorly soluble drug as predicted by the Noyes–Whitney and Ostwald–Freundlich principles, which usually leads to increasing bioavailability [19]; (ii) nanocrystalline particles require no solubilizing chemicals, therefore, it's possible to achieve high drug loading [20] and (iii) nanosized particles may lead to better antitumor efficacy *via* the enhanced permeation and retention effect, which is associated with extravasation and retention of particles in the vicinity of the tumor [21]. Given these advantages, there has been increasing interest in formulating anticancer drugs into nanosuspension formulations.

Techniques to produce nanosized crystalline particles can be categorized into top-down technologies such as milling and high pressure homogenization, and bottom-up methods such as precipitation and self-assembly [22–26]. Commonly used top-down methods have limitations such as the need for repeated milling cycles, as well as the potential for contamination from erosion of milling materials. High pressure homogenization in particular requires a relatively high number of cycles to achieve sufficient particle size reduction, which increases cost and risk of contamination and product degradation. The precipitation method involves dissolving the drug in a solvent and then adding it to a non-solvent, which leads to the production of a finely dispersed, precipitated drug. Compared to top-down techniques, a major advantage of the precipitation method is its relative simplicity and low cost. However, since solvents are used in this process, solvent choice and removal are important issues to take into consideration. Furthermore, the size of the nanocrystals can be hard to control. The stability of the nanocrystals is a major concern; particle aggregation and growth can occur and should be prevented [27].

To stabilize nanocrystal formulations, typical strategy includes the addition of hydrophilic polymers and/or surfactants to the nanosuspensions. Stabilization is achieved through repulsion by either steric or electrostatic hindrance, whereby these polymers or surfactants adsorbed onto the surface of the nanocrystals and/or alter the dielectric constant of the liquid environment. Commonly used stabilizers include semi-synthetic non-ionic polymer such as hydroxypropyl methylcellulose (HPMC), synthetic linear polymers such as polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG 400), synthetic copolymers such as Pluronic® F127 and F68, ionic surfactants such as sodium dodecyl sulfate (SDS) and non-ionic surfactants such as polysorbate esters Tween® 20 and Tween® 80 [28].

Another possible strategy is the use of proteins as stabilizers. In particular, proteins found naturally in the body, such as serum proteins, present an attractive alternative to the traditional stabilizers. Proteins have been widely used in the past as emulsifiers that help to stabilize oil-in-water emulsions [29]. Theoretically, serum proteins, such as albumin, may also provide a stabilizing effect to nanocrystals because they have been known to adsorb onto hydrophobic surfaces with a certain degree of binding affinity and therefore may provide steric hindrance to nanocrystal aggregation and growth [30,31]. In preliminary studies, it was found that the presence of serum in a drug nanosuspension resulted in effective inhibition of particle size increase during subsequent centrifugation and drying procedures compared to the absence of serum in nanosuspension (data not shown). The presence of serum was also found to inhibit aggregation of nanocrystals in an aqueous buffer environment. Therefore, it seems that serum proteins may be likely candidates for stabilizing nanosuspensions. In addition to stabilizing effects, a potential advantage of using serum proteins over traditional polymer or surfactant-based stabilizers is the ability to bind certain membrane proteins that are often present in tumorous cells [32]. The ability to bind receptor proteins present in tumor cell membranes is a property that is particularly valuable in antitumor treatments since it may facilitate targeting of anticancer drugs to the tumor site [33].

The present study describes the development and evaluation of paclitaxel nanosuspension formulations with serum protein as a stabilizer. Serum protein was successfully fractionated into different fractions and analyzed for maximal stabilizing effect. The major components of the most effective fraction (as determined by SDS-PAGE) were then analyzed further as potential stabilizers for the paclitaxel nanosuspension formulation. To the authors' knowledge, the formulation described herein is a novel approach to the development of nanoparticle-based antitumor treatment in the sense that serum protein was used as a stabilizer in a non-covalent manner. Human KB nasopharyngeal epidermal carcinoma cells and SKOV-3 ovarian cancer cell models were used to assess the *in vitro* antitumor efficacy of the formulation. The data obtained from KB cells were compared to data from mice models to assess the *in vivo* performance of the paclitaxel nanosuspension formulation.

2. Materials and methods

2.1. Preparation of paclitaxel nanocrystals

Paclitaxel (PTX) was supplied by Samyang Genex Corporation (Daejeon, Korea). Nanocrystals were prepared by an antisolvent precipitation process supplemented by sonication. In brief, 1 ml solution of PTX was injected into deionized water with or without polymers or surfactants at 4 °C under rapid stirring (1200 rpm) and intense sonication (FS20D Bath Sonicator, Fisher Scientific, Waltham, MA). The solvents evaluated were methanol, ethanol, methylene chloride (DCM), ethyl acetate (EA) and dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO). The polymers and surfactants were chosen from HPMC (Hercules Inc., Wilmington, DE), PVP (Dow Chemical Company, Midland, MI), PEG 400 (Sigma Aldrich, St. Louis, MO), Pluronic F127 and F68 (BASF, Florham Park, NJ), SDS (Sigma Aldrich, St. Louis, MO), Tween 20 and Tween 80 (Sigma Aldrich, St. Louis, MO). Processing conditions (solvent-to-antisolvent ratio, stirring speed, mixing time) were evaluated for their ability to produce stable nanosized particles less than 300 nm within 20 min of processing. A detailed flow chart of how the final process parameters were optimized is presented in Fig. 1.

2.2. Preparation of formulation

2.2.1. Serum protein fractionation

Human serum (type AB, male, Sigma Aldrich, St Louis, MO) was separated into several fractions according to a modified cold ethanol plasma-protein precipitation process [34,35]. In brief, three stock solutions were prepared: 4 M sodium acetate buffer, 10 M acetic acid and 53.3% (v/v) ethanol–water mixture were prepared by standard practices. Each fraction of serum proteins was obtained by carefully controlling the ionic strength, pH and polarity of the processing buffer environment. The ionic strength, pH and polarity of buffers were controlled by varying composition of the three stock solutions from above. Each fraction was separated from the rest by centrifugation at 3500 $\times g$ for 10 min. Serum proteins were separated using the procedure described in Fig. 1 into a total of 4 fractions and freeze dried. The fractions were stored at −20 °C until further use.

2.2.2. SDS-polyacrylamide gel electrophoresis

The serum protein fractions were characterized for their composition using SDS-PAGE by standard established procedures. Polyacrylamide gels composed of 10% stacking and 5% resolving gel were prepared. After electrophoresis, the gels were stained by Coomassie Blue and then de-stained with methanol and glacial acetic acid. The molecular weight of the protein bands was determined by electrophoresis of a standard molecular weight marker protein (Bio-Rad, Hercules, CA).

2.2.3. Formulation development

PTX nanocrystals were prepared according to procedures outlined previously in this manuscript. A certain amount of PTX nanocrystals was suspended in deionized water and added to a solution of serum protein fractions 1–4, serum protein human serum albumin (HSA), transferrin (Trf) or immunoglobulin G (IgG) (Sigma-Aldrich, St Louis,