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Nanocrystal formulations of a poorly soluble drug. 1. *In vitro* characterization of stability, stabilizer adsorption and uptake in liver cells

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ABSTRACT

In the present work, milled nanocrystals of a poorly soluble compound using different stabilizers were prepared and characterized. The aim of the study was to evaluate a fundamental set of properties of the formulations prior to i.v. injection of the particles. Two polyethylene oxide containing stabilizers; (distearoyl phosphatidylethanol amine (DSPE)) –PEG2000 and the triblock copolymer Pluronic F127, were investigated, with and without polyvinylpyrrolidone K30/Aerosol OT (PVP/AOT) present. The solubility in water was around 10 nM for the compound, measured from nanocrystals, but 1000 times higher in 4% human serum albumin. The particles were physically stable during the time investigated. The zeta potential was around −30 and −10 mV for DSPE-PEG2000 and Pluronic F127 stabilized particles, respectively, at the conditions selected. The dissolution rate was similar for all four formulations and similar to the theoretically predicted rate. Critical micelle concentrations were determined as 56 nM and 1.4 μM for DSPE-PEG2000 and Pluronic F127, respectively. The adsorption isotherms for the PEG lipid showed a maximum adsorbed amount of about 1.3 mg/m^2, with and without PVP/AOT. Pluronic F127 showed a higher maximum amount adsorbed, at around 3.1 mg/m^2, and marginally lower with PVP/AOT present. Calculated data showed that the layer of Pluronic F127 was thicker than the corresponding DSPE-PEG2000 layer. The total amount of particles distributed mainly to the liver, and the hepatocellular distribution *in vitro* (Liver sinusoidal endothelial cells and Kupffer cells), differed depending on the stabilizing mixture on the particles. Overall, DSPE-PEG2000 stabilized nanocrystals (with PVP/AOT) accumulated to a larger degree in the liver compared to particles with Pluronic F127 on the surface. A theoretical model was developed to interpret *in vivo* pharmacokinetic profiles, explaining the balance between dissolution and liver uptake. With the present, fundamental data of the nanocrystal formulations, the platform for forthcoming *in vivo* studies was settled.

1. Introduction

Nanosuspensions of milled nanocrystals are defined as colloidal dispersions with particle sizes below 1 μm, and preferably below 300 nm, enabling intravenous (i.v.) drug delivery. The small size and large surface area of the particles result in increased dissolution rate compared with larger particles of compounds which are practically insoluble in water. An additional advantage is the increased drug load resulting from milled nanocrystals compared with other drug delivery systems such as liposomes and micelles. Another advantage is reduced toxicity and adverse events compared to formulations using significant amounts of different additives. A nanocrystal formulation has three basic ingredients; drug, stabilizer(s) and dispersion medium. The medium is usually water and the stabilizers are surface active agents and/or polymers that adsorb at the surface of the drug particle. Normally, a content of less than 1% (w/w) stabilizers is enough to achieve good stability. Ionic surfactants stabilize nanosuspensions via electrostatic repulsion, while polymers and non-ionic surfactants facilitate nanosuspension stability via steric

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repulsion. Stabilizers commonly used include polymers (like polyvinylpyrrolidone (PVP) (van Eerdenbrugh et al., 2008) and hydroxypropyl cellulose (HPC) (Singh et al., 2011), ionic surfactants (like sodium dodecyl sulphate (SDS) (Singh et al., 2011) and non-ionic surfactants (like pluronics (Sharma and Garg, 2010)). The choice of stabilizers plays a crucial role in the preparation, stability, pharmacokinetic and pharmacodynamic outcome of nanocrystal therapeutics. Optimal stabilization of one specific drug does not necessarily stabilize another compound (Ito et al., 2016). Different formulation approaches, as well as differences in the surface characteristics of the crystals, imposes different requirements on the stabilizers. In addition to the described ability of the stabilizers to stabilize the particles, there is a primary interest to decrease the uptake of particles by the liver and subsequently prolong the residence time in blood to improve the *in vivo* performance of the compound.

A frequently used approach to avoid liver uptake, is to attach or conjugate different modifications of polyethylene glycol (PEG) to molecules or delivery systems. The term PEG-ylation is used to summarize modifications of therapeutic molecules and carriers with PEG. PEG-ylation has mainly been used for proteins, peptides, enzymes and small molecules (Veronese, 2001; Jevsevar et al., 2010; Li et al., 2013; Hamley, 2014), but also for drug delivery systems like liposomes (Lasic and Papahadjopoulos, 1995; Immordino et al., 2006; Barenholz, 2012), micelles (Ashok et al., 2004) and polymeric nanocarriers (Gagliardi, 2015; Prabhu et al., 2015). Regarding liposomes and micelles, PEG-ylation involves PEG conjugated phospholipids like DSPE-PEGs of different molecular weights incorporated in the lipid layers. In this case the hydrophobic part resides inside the particle or membrane and the hydrophilic PEG head orients toward the aqueous phase. PEG-ylation makes the surface hydrophilic and is supposed to hinder protein interaction (Immordino et al., 2006; Barenholz, 2012) and reduce liver uptake depending on molecular weight, structure and concentration/density of PEG chains on the particle surface. An alternative PEG structure is for instance triblock copolymers of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO), i.e. different kinds of poloxamers. At hydrophobic surfaces these polymers are expected to adsorb with the PO blocks located in the vicinity of the surface, whereas the PEG chains should protrude towards the bulk solution. The stabilizer Pluronic F127 (also named Poloxamer 407) is one of the least toxic commercially available block copolymers (Xiong et al., 2005). Nanoparticles can be PEG-ylated, or modified, in different ways. There are physical approaches for PEG-ylation on manufactured nanoparticles (including the DSPE-PEG approach decribed above), covalent conjugation of PEG to functional groups on the particle surface and preparation of the particles with PEGs present. One critical aspect when using physical modifications of the surface is what happens to the stabilization upon dilution in blood. The stabilizers are not covalently attached to the surface, but rather bound by physical interactions. There are surprisingly few studies and discussions about dilution effects in the literature. An *in vitro* study evaluated the plasma compatibility of a wet milled nanocrystal formulation under conditions that mimic an i.v. injection (Aramwit et al., 2006). The authors concluded that particle aggregation was the main source of nanosuspension instability when diluted. In another *in vitro* study, the stabilizer Pluronic F127 was investigated upon dilution of nanocrystals of paclitaxel (Deng et al., 2010). The authors found that particle size increased upon dilution e.g. due to dilution effects but, speculatively, also due to less Pluronic adsorbed on the surface at 37 °C compared to room temperature. The question was also addressed in a review about the historical and scientific perspectives on the development of Doxil®, the first FDA-approved nano-liposome formulation (Barenholz, 2012). During development of that formulation it was demonstrated that the discrepancy between successful therapeutic efficacy in mice and the failure in human studies during slow infusion was the result of the very large differences in plasma volumes (about 1 mL in mouse and >3500 mL in human). One way to investigate the physical adsorption and desorption of the stabilizers is to perform adsorption isotherms (Smith et al., 1996). An adsorption isotherm describes the equilibrium between the adsorbed amount of a stabilizer at the surface of the particle with the remaining stabilizer in the surrounding solution.

To determine the level of protection against overall hepatic uptake, and/or the distribution between cells when accumulation occurs, an *in vitro* assay was developed. In addition to being the intermediary metabolic hub of the body, and synthesizing most of the blood proteins, a major task of the liver is to scavenge blood borne material that is incompatible with homeostasis. Whereas a large number of small waste metabolites are disposed of by glomerular filtration, larger molecules and particulate waste material are mainly taken up in the liver and there degraded (Sørensen et al., 2015). This hepatic uptake is carried out chiefly by cells lining the blood capillaries of this organ, called sinusoids. The hepatic sinusoids are numerous, and place the liver among the most highly vascularized organs in the body (Greenway and Stark, 1971). With as much as 25% of cardiac output passing through the hepatic sinusoids, it is conceivable that the cells lining these capillaries are effectively exposed to the contents of the blood at any time. Furthermore, these cells are equipped with an extremely high ability to recognize and endocytose an array of macromolecules and particles of nano- and micro size, which makes these cells the most important cellular site in the body with regard to removing from the blood modified own macromolecules and cells, and macromolecules and particles of unphysiological origin. The two most important cell types responsible for the scavenger activity of the liver sinusoids are Kupffer cells (KCs) and liver sinusoidal endothelial cells (LSECs) (Sørensen et al., 2012). The KCs represent the largest population of macrophages in the body and are mainly responsible for the clearance of large particles (>200 nm) by phagocytosis, whereas the LSECs are mainly responsible for blood clearance of smaller particles (<200 nm) and macromolecules by clathrin mediated endocytosis (Elvevold et al., 2008). This dual-cell principle of waste clearance from the circulation is a relatively recent discovery (Sørensen et al., 2012), and the older concept of the reticuloendothelial system (RES), which is most commonly but incorrectly associated with macrophages only, should be abandoned. In fact, to comprehend the mechanism of hepatic blood clearance both KCs and LSECs must be included.

In the present work, nanocrystal formulations of Compound P (Fig. 1) were developed, characterized and selected as model formulations for a poorly water soluble compound. The present

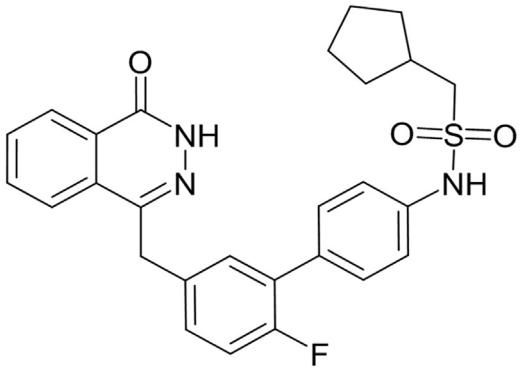

Fig. 1. The molecular structure of Compound P.