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Development of stabilized Paclitaxel nanocrystals: *In-vitro* and *in-vivo* efficacy studies

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ABSTRACT

Objective: The aim of the study was to develop stable Paclitaxel nanocrystals (PTX/NCs) for enhanced oral delivery of Paclitaxel (PTX) by circumventing its difficult solubilization properties and rapid metabolism.
Methods: Preparation of nanocrystals (NCs) was carried out using high pressure homogenizer (Microfluidizer™) without using any organic solvent. Effect of various process and formulation parameters on development and stability of nanocrystals (NCs) were investigated. Particle characteristics, stability studies, *in-vitro* cellular studies and oral pharmacokinetics in male Wistar rats were examined.
Results: It was found that different stabilizer used had different effect on size reduction and stability. Surfactants (Tween 80) and low molecular weight synthetic polymer sodium poly styrene sulfonate (PSS) found more suitable and efficient compared to high molecular weight polymers glycol chitosan (GC) and sodium alginate (SA). *In-vitro* cytotoxicity and cell cycle arrest studies on MCF7 and MDA-MB breast cancer cell lines revealed that PTX/NCs retained the activity even after processing at high pressure and also NCs were more potent and efficacious than PTX solution. The oral *in-vivo* pharmacokinetic studies demonstrated that PTX/NCs exhibit significant increase in AUC_{0-t}, C_{max}, MRT and decrease in T_{max}, compared to plain PTX crystals. The increase in AUC was almost 9–10 fold compared to plain PTX crystals.
Conclusion: Altogether study showed that PTX/NC can be a clinically relevant drug delivery system for oral chemotherapy as it can remarkably increases the pharmacological effect by increasing oral bioavailability.

1. Introduction

Cancer is one of the leading causes of death worldwide and still chemotherapy is indispensable during clinical treatment. However, most of the anticancer agents are poorly soluble in aqueous phase and sometimes in organic phase as well which results in their low oral bioavailability. Paclitaxel (PTX) is a diterpenoid isolated form *Taxus brevifolia*, is a first line chemotherapeutic agent against tumor treatment with high lipophilicity (log *P* 3.20) and low water solubility <0.1 μg/ml). Although being the most promising drug against tumor the clinical utility of PTX is constrained due to its limited solubility in water and poor permeability across GIT membrane (Agüeros et al., 2010). Currently, it is typically administered as intravenous infusion in a mixture of Cremophor EL™ (polyoxyethylated castor oil) and alcohol (1:1 by weight) and marketed as Taxol® or Paxene®. Cremophor is biologically and pharmacologically active solvent which cause number of side effects, including hypersensitivity, nephrotoxicity, neurotoxicity, hyperlipidaemia as well as alteration of PTX pharmacokinetics (Bayindir and Yuksel, 2010; Gelderblom et al., 2001; Weiss et al., 1990). Abraxane™ is another protein bound FDA approved formulation of PTX, though devoid of Cremophor EL™, it is an injectable formulation (Pandita et al., 2011) and expensive. It involves the use of organic solvents during preparation. Oral delivery is always the preferred choice for drug administration over i.v delivery, because of its better patient compliance, reduction in administration cost and usefulness in chronic treatment regimens. Oral chemotherapy is an important step for "chemotherapy at home" (Feng et al., 2009; Thanki et al., 2013). However oral delivery of PTX is a challenging task because of several reasons which includes its low water solubility, efflux by transporter P-glycoprotein and its affinity for intestinal and liver cytochrome P450 metabolic enzymes (Malingré et al., 2001; Sparreboom et al., 1997). CYP3A4, s a well known enzyme co-located with P-gp in the intestine, is primarily responsible for the metabolism of PTX to C3′-hydroxypaclitaxel.

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Several strategies for its oral administration have already been explored such as co-administration of PTX with P-gp and CYP450 inhibitors. However none of them reached into the market. Infact potential PTX analogues BMS-275183 (Broker et al., 2007) and ortotaxel (Brooks et al., 2003) were developed which showed 50% oral bioavailability but in both cases significant toxicological profile was observed. Nano-carriers are promising tool for oral chemotherapy. They can enhance the oral bioavailability either by enhancing the solubility, by enhanced adhesion to GI membrane or by direct transporting the drug to systemic circulation across the GIT membrane escaping the P-gp efflux, gut metabolism and liver metabolism. But most commonly used polymeric or lipidic nano-carriers suffer the limitation of low drug loading and industrial scalability. Several other PTX dosage forms developed earlier, specifically pro-drugs designed to improve water solubility, suffers the limitation of chemical degradation in aqueous solution at neutral pH (Cavallaro et al., 2004; Li et al., 1998). Nanocrystals (NCs) are type of nano-carriers consisting of sub-micron size pure drug crystals stabilized by minimum amount of suitable stabilizer (Gao et al., 2008) and have recently gained high attention of researchers for the oral delivery of drugs (Gao et al., 2013). They are known to enhance the oral bioavailability of drugs by enhancing the dissolution rate, saturation solubility and increased adhesion to the mucosal surface (Muller et al., 2011). Besides they improve the stability of chemically labile drugs in comparison to solution (Teeranachaideekul et al., 2008). Moreover, very recently Fu et al. reported that these nanocarriers can be directly absorbed in systemic circulation via mesenteric lymphatic transport facilitated by endocytosis and thus can bypass the gut and liver metabolism (Fu et al., 2013).

Several NCs formulations of antitumor drugs such as Campothecin, Bicalutamide has shown marked improvement in their oral absorption (Mandpe and Pokharkar, 2013; Zhang et al., 2011) while, several other drug NC formulations are already in the market i.e., Rapamune®, Emend®, TriCor®, Megace® ES, Triglide®, Avinza®, Facalin® XR, Ritalin® LA and Zanaflex Capsules (Singare et al., 2010). Development of Paclitaxel nanocrystals PTX/NCs for the oral delivery can be promising approach, first it can circumvent disadvantages of classical formulation Taxol® by avoiding use of Cremophor®, second high drug loading and industrial scalability can be achieved which is a major limitation of other nano-carriers (Fu et al., 2013; Mandpe and Pokharkar, 2013). The only issue with NCs is their physical stability, since NCs consists of very large surface area compared to the conventional suspensions they requires sufficient amount of additives to ensure sufficient stabilization (Cerdeira et al., 2010). Although both surfactants and polymeric stabilizers can be used but a critical evaluation of the type and the concentration of stabilizers is required for successful production.

There are others groups who have also worked on the development of PTX/NCs and most of them are for i.v delivery. However, these groups have developed nano-crystals by bottom-up method which involves the use of organic solvents like chloroform or ethanol, further the technique is not industrially scalable. Moreover, the ratio of drug to that of the excipient is very low and physical stability requires more investigation. The aim of the present work is to develop stable PTX/NCs for oral delivery without using any organic solvents by high pressure homogenizer (Microfluidizer™) with minimum amount of stabilizers. Microfluidization or high pressure homogenization is an industrially feasible technique in which size reduction is based upon the particle collision and shear forces generated when the dispersion is subjected through the interaction chamber. Pharmaceutical characterization of NCs was performed in terms of particle size analysis, dissolution test, scanning electron microscopy (SEM) imaging, atomic force microscopy AFM and X-ray diffraction (XRD). The *in-vitro* efficacy studies were carried out to ensure that the PTX molecules retain the activity even processing at such high pressure. The efficacy and suitability (both *in-vitro* and *in-vivo*) of prototype formulation for delivery of PTX was assessed by cytotoxicity studies and oral bioavailability studies.

2. Materials and methods

2.1. Materials

The drug PTX (>99% purity) was obtained as gift sample from Dabur Research Foundation (Ghaziabad, India). Sodium poly styrene sulfonate (PSS), Glycol Chitosan (GC), sodium alginate (SA) and Mannitol was procured from Sigma-Aldrich (Missouri, USA). Ultra purified water was supplied from a MilliQ Plus system, Millipore. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], dimethyl sulfoxide (DMSO). Ten percent heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin, 100 μg/mL of streptomycin, Dulbecco's modified Eagle's medium (DMEM) and supplements were also purchased from Sigma Aldrich (Missouri, USA). Methanol and acetonitrile HPLC grade were from Sigma Aldrich (Missouri, USA). All other chemicals and reagents were of LR grade and used without additional purification. Tissue culture flask six well and 96 well plates were from Greiner Bio One (Frickenhausen, Germany).

2.2. Preparation of PTX/NCs

PTX/NCs were prepared using Microfluidizer (MP-110) as homogenization device. PTX (50 mg) was dispersed in 50 ml aqueous solution of various stabilizers separately (PSS, GC, SA and Tween 80) in different concentrations as shown in Table 1. After dispersion pre-milling was carried out using Ultraturrax (IKA Germany high speed homogenizer) at 10,000 rpm for 5 min followed by milling at increasing pressure (2 cycles at 300 bar, 2 cycles at 500 bars, and 1 cycle at 1000 bar) in Microfluidizer. This microsuspension was then subjected to high pressure homogenizer (Microfluidizer™) at 1500 bar for 20 homogenization cycles to obtain the nano-sized drug particles. Samples for characterization were collected after pre-milling, as well as after 1, 5, 10, 15 and 20 cycles of homogenization. Since this whole process causes the increase in temperature, all operations were carried out using an heat exchanger with sample temperature maintained at 4 °C. The NCs were then lyophilized using mannitol as cryoprotectant to obtain the dry powder. First the samples were frozen in round bottom flask using liquid nitrogen to form a thin layer on the surface and then transferred to freeze dryer (Labconco Labconco Corporation, USA). Sample temperature was maintained at −45 °C for 24 h with secondary cycle of 5 h at 25 °C. Mannitol (10% w/w, Mannitol/drug) was added into nanocrystals as cryoprotectant prior to lyophilization. Dried samples were found re-dispersible on manual shaking and are used for further characterization.

2.3. Characterization of PTX/NCs

2.3.1. Particle size, poly-dispersity index (PDI) and zeta potential (ZP) analysis

The mean particle size (z-average) and polydispersity index (PDI) under different homogenization conditions were determined

Table 1
Composition of different NCs formulations.

Formulation	% w/v Stabilizer used for 50 mg of PTX		
PTX/NC-PSS	0.025	0.05	0.1
PTX/NC-T80	0.025	0.05	0.1
PTX/NC-GC	0.1	0.25	0.5
PTX/NC-SA	0.1	0.25	0.5