

# Development of a Nanocrystalline Paclitaxel Formulation for Hipec Treatment

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## ABSTRACT

**Purpose** To develop a nanocrystalline paclitaxel formulation with a high paclitaxel-to-stabilizer ratio which can be used for hyperthermic intraperitoneal chemotherapy (HIPEC).

**Methods** Paclitaxel (PTX) nanocrystals were prepared *via* wet milling using Pluronic F127<sup>®</sup> as stabilizer. The suitability of paclitaxel nanosuspensions for HIPEC treatment was evaluated by analyzing the cytotoxicity of both stabilizer and formulation, and by determining the maximum tolerated dose (MTD) and bioavailability. The effect on tumor growth was evaluated by magnetic resonance imaging (MRI) at day 7 and 14 after HIPEC treatment in rats with peritoneal carcinomatosis of ovarian origin.

**Results** Monodisperse nanosuspensions ( $\pm 400$  nm) were developed using Pluronic F127<sup>®</sup> as single additive. The cytotoxicity and MTD of this nanocrystalline formulation was similar compared to Taxol<sup>®</sup>, while its bioavailability was higher. MRI data after HIPEC treatment with a PTX nanocrystalline suspension showed a significant reduction of tumor volume compared to the non-treated group. Although no significant differences on tumor volume were observed between Taxol<sup>®</sup> and the nanosuspension, the rats treated with the nanosuspension recovered faster following the HIPEC procedure.

**Conclusion** Nanosuspensions with a high paclitaxel-to-stabilizer ratio are of interest for the treatment of peritoneal carcinomatosis of ovarian origin *via* HIPEC.

**KEY WORDS** hyperthermic intraperitoneal chemotherapy · nanocrystal · ovarian cancer · paclitaxel · wet milling

## ABBREVIATIONS

HIPEC	hyperthermic intraperitoneal chemotherapy
MTD	maximum tolerated dose
PEO	polyethylene oxide
Plu F127	Pluronic F127 <sup>®</sup>
Plu F68	Pluronic F68 <sup>®</sup>
PPO	polypropylene oxide
PTX	paclitaxel
TGD	tumor growth delay

## INTRODUCTION

Ovarian cancer is the fifth most common cancer affecting European women (1). Once the tumor starts growing in the ovary, spread of cancer cells throughout the abdominal-pelvic cavity occurs very early in the development of the disease (2). The standard therapy for patients with peritoneal carcinomatosis of ovarian origin is initial cytoreductive surgery followed by intravenous platinum-taxane chemotherapy (3,4). Lately, this standard therapy has been modified as alternative

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treatments have been developed. A treatment following cytoreductive surgery is hyperthermic intraperitoneal chemotherapy (HIPEC). Administration of chemotherapy intraperitoneally under hyperthermic conditions may improve the mean overall survival of patients with advanced ovarian cancer from 19.0 to 76.1 months (5). Paclitaxel is a suitable molecule for HIPEC treatment as it has a high peritoneal/plasma concentration ratio (>1000) and a significant first pass effect (6). However, paclitaxel is not commonly used for HIPEC treatment because of the side effects caused by Cremophor EL<sup>®</sup> used as solubilizer in the commercially available formulation Taxol<sup>®</sup>, like abdominal pain and life-threatening hypersensitivity reactions (7). Due to these side effects a lot of research is done, developing new paclitaxel formulations without resorting to toxic excipients to improve the solubility of paclitaxel.

As size reduction is an efficient method to improve the performance of poorly soluble drugs (the increased surface area of smaller particles enhances dissolution rate and bioavailability based on the Noyes-Whitney equation), wet milling was used to obtain a nanosuspension of paclitaxel. While top-down (particle size reduction) and bottom-up (precipitation method) techniques can be used to manufacture nanoparticles (8), wet milling is a typical top-down method using milling beads to grind the particles. This procedure is known as an efficient method to prepare nanoparticles with easy scale-up and limited batch-to-batch variability when the method is optimized. Contamination of the final product due to erosion of the milling beads is a main concern, while changes in physical form or amorphization can also be an issue during wet milling (9). As breakage of drug crystals into nanoparticles significantly increases particle surface area, the higher Gibbs free energy creates a thermodynamically unstable nanosuspension, and proper selection of a stabilizer is required during the preparation of the nanosuspension to prevent agglomeration or crystal growth (due to Ostwald ripening) of the nanoparticles (10).

In this study, the wet milling technique is applied to obtain a paclitaxel nanosuspension stabilized with a surfactant (polyethylene oxide-polypropylene oxide block copolymers, Pluronic F68<sup>®</sup> and Pluronic F127<sup>®</sup>). Pluronic-stabilized paclitaxel nanocrystals have already been formulated, but were characterized by a low drug-to-stabilizer ratio: Lui *et al.* required at least a paclitaxel/Pluronic F127<sup>®</sup> ratio of 1/5, as at lower stabilizer concentrations stable nanocrystals could not be formed (11). In order to maximize the drug concentration at the delivery site, which is one of the challenges of HIPEC therapy (12), the stabilizer content in the nanocrystalline formulation was minimized in our study. After characterization of the nanocrystals processed *via* the wet milling technique, the feasibility of the nanosuspension for HIPEC treatment was assessed (in comparison with Taxol<sup>®</sup>) by evaluating *in vitro* cytotoxicity of the excipients as well as the formulation on an ovarian cancer cell

line, while the toxicity, bioavailability and effect on tumor growth were tested in a rat model.

## MATERIALS AND METHODS

### Materials

Paclitaxel (PTX) was purchased from Enzo Life Sciences (Zandhoven, Belgium). Polyethylene oxide-polypropylene oxide (PEO-PPO) block copolymers, Pluronic F68<sup>®</sup> and pluronic F127<sup>®</sup>, were obtained from BASF (Ludwigshafen, Germany), Taxol<sup>®</sup> from Bristol-Myers Squibb (Brussels, Belgium) and Cremophor EL<sup>®</sup> from Alpha Pharma (Waregem, Belgium).

### Preparation of Paclitaxel Nanocrystals

Paclitaxel nanosuspensions were prepared by a wet milling technique using two different stabilizers (Pluronic F68<sup>®</sup> and pluronic F127<sup>®</sup>) in three PTX/stabilizer ratios (2/1, 4/1 and 8/1). After dissolving the stabilizer in a 20 ml vial containing 5 ml of 0.9 % NaCl, paclitaxel powder (50 or 100 mg) was dispersed in this aqueous phase. Zirconium oxide beads (amount 30 g, diameter 0.5 mm) were added to the suspension as milling pearls. The vials were placed on a roller-mill (Peira, Beerse, Belgium) and grinding was performed at 150 rpm for 24 or 60 h. After milling the nanoparticles were separated from the grinding pearls by sieving.

For solid state characterization of the PTX nanocrystals, the nanosuspension was freeze dried for 24 h at  $-50^{\circ}\text{C}$  and 1 mbar.

### Nanocrystal Characterization

The mean particle size and polydispersity index (PI) of the nanosuspensions was determined by photon correlation spectroscopy, using a Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). Prior to analysis, the nanosuspensions were diluted with distilled water and were analyzed at room temperature.

The morphology of the freeze dried drug particles was observed under a scanning electron microscope (SEM) (JSM 5600 LV, Jeol, Tokyo, Japan) after coating the powder particles with platinum using a sputtering equipment (Auto Fine Coater, JFC-1300, Jeol, Tokyo, Japan).

Thermal properties of the freeze dried samples were analyzed by differential scanning calorimetry (DSC Q2000, TA instruments, Leatherhead, UK). Samples were placed in sealed aluminum pans, and evaluated over a temperature range from  $-20$  to  $190^{\circ}\text{C}$  with a heating rate of  $10^{\circ}\text{C}/\text{min}$ . Pure drugs and physical mixtures were tested as controls. The thermal profiles were analyzed using the TA Instruments Universal Software.

## 2.4. *In vitro* cytotoxicity

The human ovarian carcinoma cell line (SKOV-3, obtained from the American Type Culture Collection) was cultured at 37°C in a 5 % CO<sub>2</sub>-containing humidified atmosphere in McCoy's medium (Invitrogen, Merelbeke, Belgium). The medium was supplemented with 10 % fetal bovine serum, penicillin, streptomycin (Invitrogen, Merelbeke, Belgium) and fungizone (Bristol Myers Squibb, Brussels, Belgium).

The cytotoxicity of PTX nanosuspensions (at a PTX/Plu F127 ratio of 4/1) and of Taxol<sup>®</sup> was tested at PTX concentrations of 0.01, 0.1, 1, 5 and 10 µg/ml, 8 wells per concentration were used. Both Taxol<sup>®</sup> and PTX nanosuspensions were diluted with 0.9 % NaCl to the appropriate concentration. In addition, the cytotoxicity of the excipients in these formulations (Pluronic F127<sup>®</sup> and Cremophor EL<sup>®</sup>) was tested using 9 concentrations: 0, 0.01, 0.1, 1, 1.5, 2, 2.5, 3 and 3.5 mg/ml.

To evaluate the cytotoxicity, 20 × 10<sup>3</sup> cells/ml were seeded in 96-well plates (Sarstedt, Newton NC, USA). After 72 h, 20 µl medium was removed and replaced by the test formulation. After incubation for 1 h at 41.5°C (*i.e.* to mimic the HIPEC procedure used during *in vivo* studies), the medium was entirely removed, cells were washed with PBS and 200 µl fresh medium was placed in each well. Afterwards, the cells were incubated for 24 and 96 h at 37°C under 5 % CO<sub>2</sub>-atmosphere. The cytotoxicity of the test formulations was determined *via* MTT assay and compared with the non-treated cells (13). Afterwards, the optical density was measured at 570 nm normalizing with a reference wavelength of 650 nm using an ELISA-plate reader (Paradigm Detection Platform, Beckman Coulter, Suarlecé, Belgium).

## *In Vivo* Testing

### HIPEC Procedure

Adult female athymic nude rats (Harlan, Horst, The Netherlands) were kept in standard housing conditions with water and food *ad libitum* and a 12 hours light/dark cycle. All animal experiments were approved by the Ethical Committee of the Faculty of Medicine, Ghent University (ECD 09/06).

After anesthetizing each rat with 3 % isoflurane (Forene<sup>®</sup>, Abbott, Belgium) a vertical incision was made along the midline in the abdominal wall muscle. The abdominal wall muscle was attached to a metal ring which was placed a few centimeters above the incision. The inlet and outlet tubing (Pumpsil<sup>®</sup>, Watson-Marlow, Zwijnaarde, Belgium) was placed in the peritoneal cavity for perfusion with the cytostatic solution over a period of 45 min. A roller pump (Watson-Marlow, Zwijnaarde, Belgium) circulated the cytostatic solution through a heat exchanger set at 41.5°C. During perfusion, the perfusate solution and body temperature of the rat were closely monitored and data was collected using E-Val<sup>®</sup> 2.10 Software

(ELLAB<sup>®</sup>, Roedovre, Denmark). After HIPEC, the cytostatic perfusate solution was removed and the incision was sutured.

### Maximum Tolerated Dose

The maximum tolerated dose (MTD) was determined for both PTX formulations: Taxol<sup>®</sup> and PTX/Plu F127 nanosuspension (ratio 4/1). The MTD was defined as the highest non-lethal dose with a maximum reduction of body weight of 10 % after 2 weeks of HIPEC treatment. The test procedure was based on the Organization for Economic Co-operation and Development (OECD) guidelines. Based on the maximum tolerated dose of Taxol<sup>®</sup> (0.24 mg/ml) determined by Bouquet *et al.* in Wag/Rij rats (13), and based on the lower body weight and the reduced immune system of the athymic nude rats used in this study, a PTX dose of 0.21 mg/ml was used as starting point. To determine MTD, the PTX concentration in the formulation was gradually increased (increments of 0.03 mg/ml) using 1 rat per concentration. When mortality occurred, 3 more rats were tested at the highest non-lethal dose to confirm MTD. MTD of both formulations was determined using HIPEC settings (*i.e.* 41.5°C during 45 min). The PTX nanosuspensions as well as Taxol<sup>®</sup> were diluted with 0.9 % NaCl to obtain the correct dose in an isotonic solution.

### Bioavailability

Blood was sampled in heparin-containing tubes *via* a catheter that was placed in the arteria carotis, at 0, 15, 30, 45, 60 and 90 min after starting the perfusion blood samples were taken. Perfusate samples were collected at 0, 15, 30 and 45 min after starting the perfusion, to ensure that an accurate dose was administered during the HIPEC procedure. After the bioavailability study the rats were euthanized. Blood samples were centrifuged immediately afterwards, and separated plasma was stored frozen at -20°C until analysis.

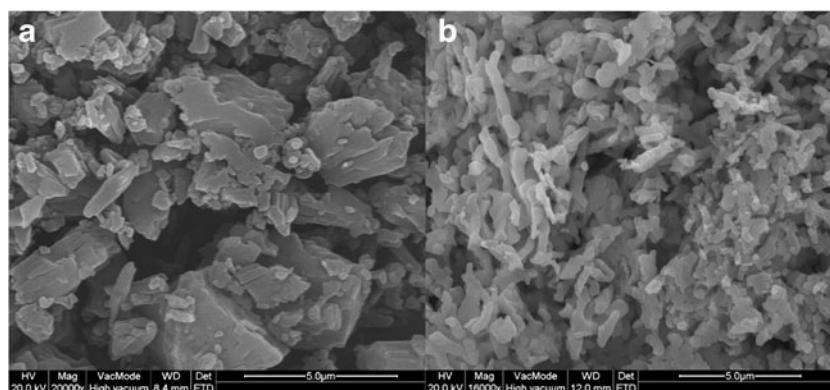
The perfusate samples were analyzed by high performance liquid chromatography (HPLC). The HPLC-system (Merck-Hitachi, Tokyo, Japan) consisted of a pump (L-6000), an integrator (D-2000), an autosampler (L-7200) with a 25 µl loop and a UV/VIS detector (L-4200). Detection was performed at 227 nm. To achieve chromatographic separation a guard column (Lichrospher<sup>®</sup> 100-RP-18, 4\*4 mm (5 µm), Merck, Darmstadt, Germany) and an analytical column (Lichrospher<sup>®</sup> 100-RP-18, 125\*4 mm (5 µm), Merck, Darmstadt, Germany) were used. The mobile phase consisted of acetonitrile (Biosolve, Valkenswaard, The Netherlands) and 0.1 % (v/v) phosphoric acid in ultrapure water (Acros Organics, Geel, Belgium) (42:58, v/v) degassed by ultrasonication under vacuum.

Frozen plasma samples, calibrator samples and QC samples were allowed to thaw at room temperature. For samples within the assay range a volume of 50 µl was transferred to an empty well of a 96-well filtration plate. Other samples

**Table 1** Mean Particle Size  $\pm$  STD (nm) and Polydispersity Index After Wet Milling (at 150 rpm) of a Paclitaxel Suspension

Stabilizer	PTX/stabilizer ratio (w/w)	amount PTX (mg)	milling time (h)	size $\pm$ STD (nm)	PI
Pluronic F68®	2/1	100	24	4057 $\pm$ 1042	0.368
	4/1	100	24	3374 $\pm$ 1731	0.421
	8/1	100	24	3208 $\pm$ 765	0.470
Pluronic F127®	2/1	100	24	417 $\pm$ 72	0.351
	4/1	100	24	462 $\pm$ 128	0.308
	8/1	100	24	812 $\pm$ 154	0.425
	2/1	100	60	420 $\pm$ 18	0.260
	4/1	100	60	440 $\pm$ 30	0.268
	8/1	100	60	462 $\pm$ 66	0.302
	2/1	50	60	325 $\pm$ 12	0.224
	4/1	50	60	307 $\pm$ 12	0.232
8/1	50	60	375 $\pm$ 21	0.287	

(samples above upper limit of quantitation) were diluted sixfold with blank rat plasma prior to the analysis. Subsequently 200  $\mu$ l of acetonitrile containing internal standard ( $C^{13}$ -paclitaxel) was added. After vacuum filtration, the filtrate was diluted with 150  $\mu$ l of water and injected onto the Waters Acquity UPLC system for analysis. After chromatographic separation on a Waters Acquity UPLC BEH C18 column (100 mm  $\times$  2.1 mm, 1.7  $\mu$ m particle size), PTX and the internal standard were detected using a Waters Quattro Ultima triple quadrupole system (Micromass Waters, Manchester, UK). The validation of the method was conducted with reference to the FDA's guidance for bioanalytical method validation. The calibration curve was constructed by least squares linear regression of the peak area ratio of PTX/internal standard against nominal concentration with a weighting of concentration<sup>-1</sup>. The measurement range of the analytical method was 2.0–500 ng/mL for PTX in rat plasma. The range was further extended up to 3  $\mu$ g/ml by appropriately diluting plasma samples prior to analysis. Total imprecision and trueness were calculated on results of repeated analysis of quality controls on different days. For all levels of the QC samples, imprecision and trueness measurements comply with the FDA guidance specifications on maximum tolerable bias and imprecision.

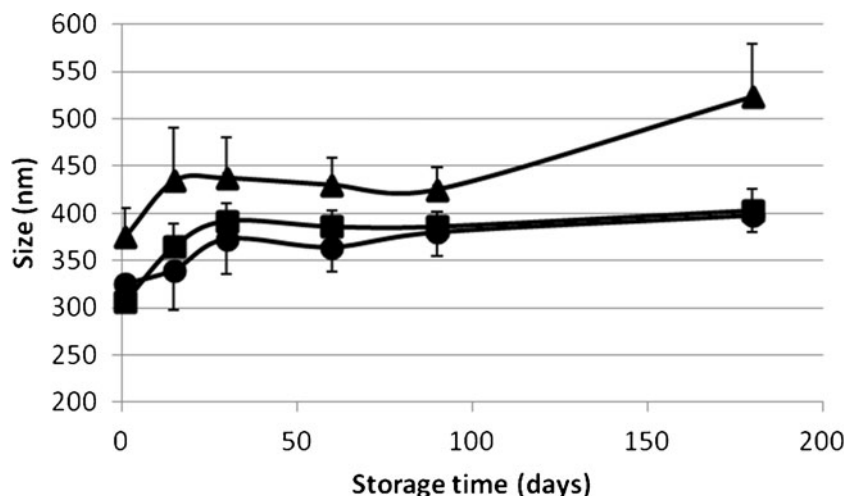
**Fig. 1** SEM images of freeze dried PTX nanocrystals, processed via wet milling (150 rpm, 60 h), using a PTX load of 50 mg per vial and a PTX/stabilizer ratio of 4/1. Pluronic® F127 was used as stabilizer.

### Tumor Growth Delay

Donor rats were injected with  $30 \times 10^6$  SKOV-3 cells between the peritoneum and the abdominal muscle. The animals received daily subcutaneous cyclosporine injections (dose: 3 mg) over a period from 3 days prior until 10 days after tumor cell injection. After 3–4 weeks, a tumor was induced, but this tumor remained localized and did not (or not completely) penetrate the peritoneum. Therefore, parts (5  $\times$  5 mm, with a thickness of 3 mm) of the tumor induced in the donor rats were transplanted on the peritoneum of acceptor rats. The acceptor rat also received daily subcutaneous cyclosporine injections (dose: 3 mg) from 3 days prior until 10 days after tumor transplantation to ensure tumor attachment. Two weeks after transplantation, the tumor had attached on the peritoneum and was sufficiently grown to perform the tumor growth delay (TGD) experiment.

The effect of the PTX formulations (Taxol® and nano-suspension) on tumor growth was evaluated via a Siemens® Trio 3 T MRI (Erlangen, Germany). Prior to the MRI scan, the rats were anaesthetized with Rompun 2% (Bayer, Diegem, Belgium) and ketamine 1000 CEVA (Ceva, Amersham, UK) using a dose of 10 mg/kg and 90 mg/kg, respectively. The rats

**Fig. 2** Particle size (mean  $\pm$  STD) of PTX nanosuspensions as a function of storage time at ambient conditions. PTX/Pluronic F127 ratio: (●) 2/1, (■) 4/1, (▲) 8/1.



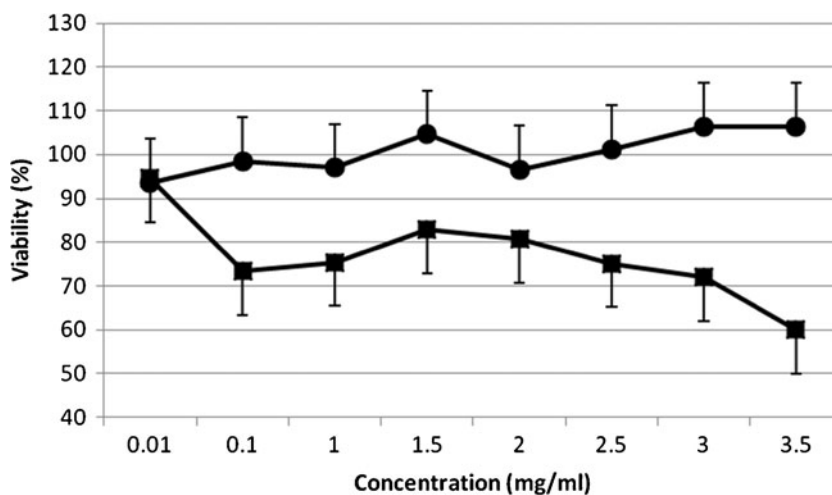
were placed prone in a (wrist) coil to measure the tumor volume. A T1-weighted 3D FLASH sequence was applied with a flip angle of  $10^\circ$ , a repetition time of 13 ms and echo time of 4.9 ms to obtain a voxel size of  $0.19 \times 0.19 \times 0.4 \text{ mm}^3$ . In order to easily locate the tumor, the rat was palpated and a vitamin B12 pellet was attached to the skin where the tumor was located. Tumor volume was calculated using PMOD software (PMOD Technologies, Adliswil, Switzerland). Rats were scanned 1 day before HIPEC treatment to measure the initial volume of the tumor. At day 0 the rats were treated with Taxol<sup>®</sup> or the nanocrystalline PTX formulation. Tumor volume was evaluated 7 and 14 days after HIPEC treatment to monitor the effect of both PTX formulations.

### Statistical Analysis

Statistical Program for Social Scientists (SPSS 19.0) was used to analyze the results.

For the bioavailability study, the pharmacokinetic parameters of both groups were compared using an unpaired sample *t*-test with a significance level of 0.05.

**Fig. 3** Viability (mean  $\pm$  STD) of the SKOV-3 cell line after application of different concentrations of Cremophor EL<sup>®</sup> and Pluronic F127<sup>®</sup> at hyperthermic conditions ( $41.5^\circ\text{C}$ ) ( $n=8$  wells per concentration). (●) Pluronic F127<sup>®</sup>, (■) Cremophor EL<sup>®</sup>.



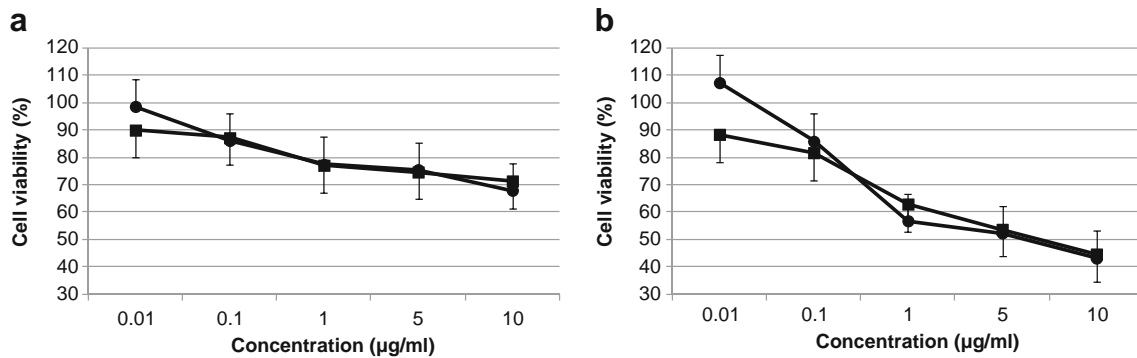
For the TGD study, data of day 0 were used as reference (100 %). The different treatment groups were compared with each other on day 7 and 14 using a one-way ANOVA with a significance level of 0.05. Bonferroni post-hoc analysis was performed for pairwise comparisons between treatment groups.

## RESULTS AND DISCUSSION

### Physico-chemical Characterization of PTX Nanocrystals

Pluronic F68<sup>®</sup> and Pluronic F127<sup>®</sup> were selected as stabilizers because these block copolymers have already been successfully used to stabilize PTX nanosuspensions (both at a higher surfactant/PTX ratio than used in this study) (11). In addition, these surfactants are known to increase the solubility of low soluble drugs, and have cytotoxicity-promoting properties as they interact with multi-drug resistance cancer tumors, resulting in drastic sensitization of





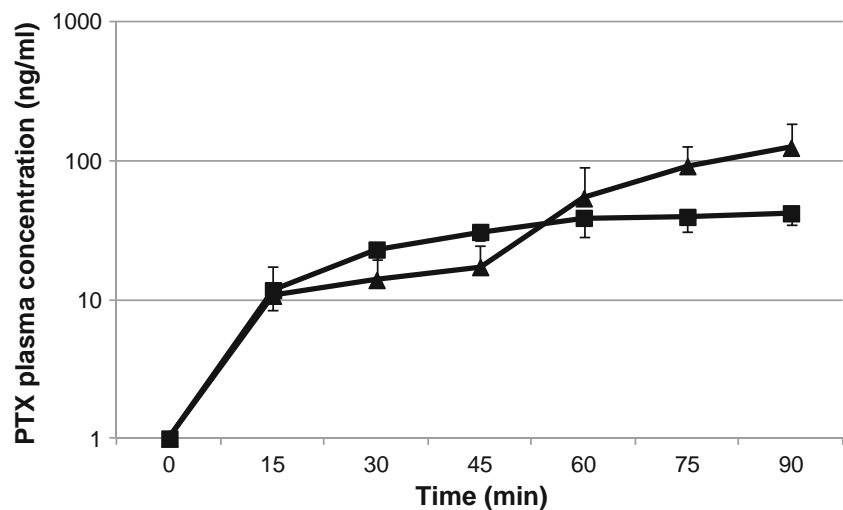
**Fig. 4** Viability (mean  $\pm$  STD) of the SKOV-3 cell line ( $n=3$  and 8 wells per concentration) after application of different PTX concentrations delivered under hyperthermic conditions (41.5°C) via (■) Taxol® and (●) PTX/Plu F127 4/1 nanosuspension. MTT after 24 h (a) and 96 h (b).

these tumors to the cytostatic drugs (14–16). A wet milling cycle during 24 h did not yield nanocrystalline PTX when Pluronic F68® was used as stabilizer (independent of the PTX/stabilizer ratio) (Table I). In contrast, Pluronic F127®-containing formulations were efficiently grinded into nanosuspensions. Although Pluronic F68® and F127® have the same basic PEO-PPO-PEO structure, they differ in the number of PEO and PPO groups. The higher molecular weight and lower hydrophilic-lipophilic balance (HLB) value of Pluronic F127® compared to Pluronic F68® allowed more interaction between the amphiphilic surfactant and the nanoparticle surface, providing sufficient steric hindrance to stabilize the nanoparticles and prevent particle agglomeration (17). However, a minimum concentration of stabilizer was required as at the lowest Pluronic F127® content (*i.e.* PTX/stabilizer ratio of 8/1) the surfactant failed to sufficiently stabilize the PTX particles. A longer milling time (60 h) not only yielded PTX nanocrystals at all PTX/Pluronic F127® ratios, it also resulted in a narrower particle size distribution as indicated by the lower polydispersity indices (PI). Reducing the PTX amount during wet milling to 50 mg improved the efficiency of the milling process as all formulations yielded a mean particle size below 400 nm, in combination with low PI values

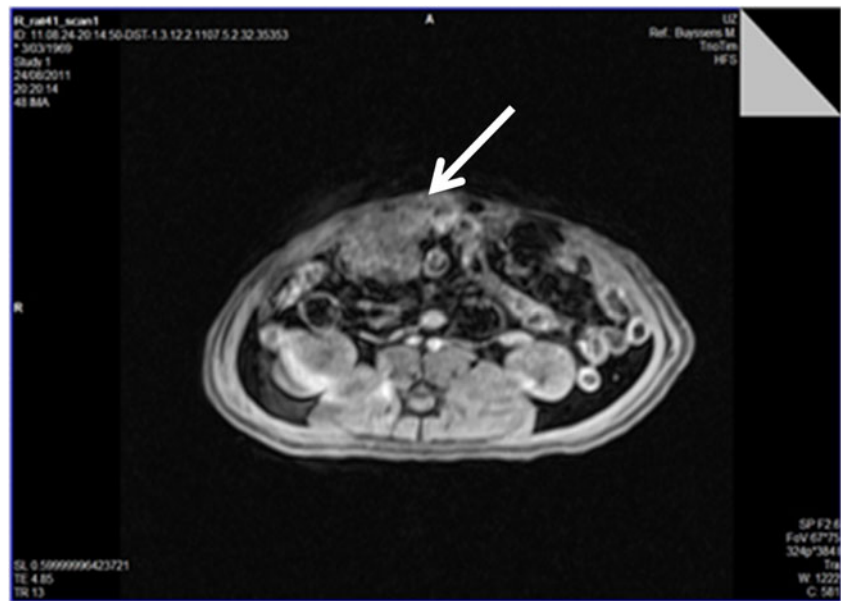
(<0.3). A further reduction of PTX load had no effect on particle size. SEM analysis showed a reduction in particle size compared with unmilled PTX (>5 µm). A size around 400 nm was obtained, which confirmed the results obtained by particle size analysis (Fig. 1). Solid state characterization by DSC of the freeze dried nanocrystalline formulation showed that the crystallinity of PTX was not affected by the wet milling process, thus avoiding possible stability issues due to crystalline-to-amorphous transitions induced by the friction generated during the high-intensity wet milling process (18).

A 6 month stability study at ambient conditions of the different PTX/Plu F127 formulations (Fig. 2) indicated that initially the particle size of all 3 formulations slightly increased. Afterwards the particle size of PTX/Plu F127 2/1 and 4/1 nanocrystals remained constant ( $\pm 400$  nm) with PI values below 0.3. In contrast, the PTX/Plu F127 8/1 formulations became polydispers (PI > 0.5) and particle agglomeration was observed. Due to these stability issues the PTX/Plu F127 8/1 formulation was not used in further experiments. As the goal of this study was to reduce the stabilizer concentration in the nanosuspensions as much as possible, the PTX/Plu F127 4/1 formulation was selected for further *in vitro* and *in vivo* experiments.

**Fig. 5** Paclitaxel plasma concentration (mean  $\pm$  STD) (ng/ml) in rats ( $n=6$ ) during and post-HIPEC treatment with a PTX concentration of 0.24 mg/ml delivered via (▲) Taxol® and (■) a PTX/Plu F127 4/1 nanosuspension.



**Fig. 6** Dorsal MRI image of a rat with a peritoneal tumor (white arrow).



### In Vitro Cytotoxicity

The cytotoxicity of the PTX formulations (Taxol<sup>®</sup> and nanosuspension) as well as the excipients (Pluronic F127<sup>®</sup>, Cremophor EL<sup>®</sup>) was tested on human ovarian carcinoma cells (SKOV-3) as ovarian cancer commonly results in peritoneal carcinomatosis which can be treated *via* HIPEC. As the main drawback to the use of Taxol<sup>®</sup> for HIPEC treatment are the side effects caused by the excipient Cremophor EL<sup>®</sup>, the cytotoxicity of Cremophor EL<sup>®</sup> and Pluronic F127<sup>®</sup> was compared in a concentration range from 0.01 to 3.5 mg/ml. After 1 h incubation, there was no reduction of cell viability at the lowest concentration (Fig. 3). However, at higher concentrations the cell viability decreased after contact with Cremophor EL<sup>®</sup> while the cells treated with Pluronic F127<sup>®</sup> were unaffected, indicating a significantly lower cytotoxicity of Pluronic F127<sup>®</sup> compared to Cremophor EL<sup>®</sup>. Despite Pluronic F127<sup>®</sup> not being cytotoxic, both PTX formulations were equipotent, as the cytotoxicity of the new nanosuspension formulation was equal to Taxol<sup>®</sup> (Fig. 4).

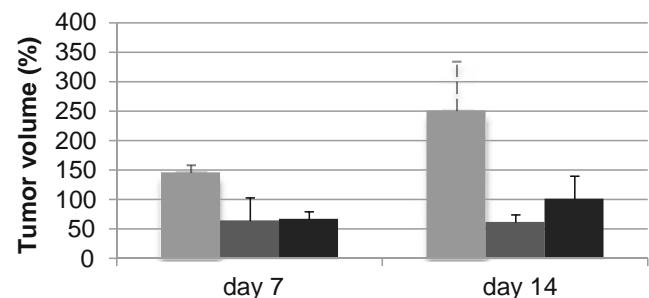
### Nanocrystalline PTX for HIPEC Treatment

The maximum tolerated dose (MTD) of both PTX formulations after HIPEC was determined by monitoring the survival rate and body weight of the rats following HIPEC treatment. While at a PTX dose of 0.21 and 0.24 mg/ml the rats regained their initial body weight after 2 weeks, a PTX concentration of 0.27 mg/ml for Taxol<sup>®</sup> and the nanosuspension resulted in mortality. Hence, 0.24 mg/ml was set as MTD for both formulations. At this concentration no significant differences were observed between both treatments based on the weight of the rats. However, rats treated with

the nanosuspension recovered faster compared to the group treated with Taxol<sup>®</sup> as they already regained their initial body weight 5 days after HIPEC treatment, highlighting the advantage of using the non-cytotoxic Pluronic F127<sup>®</sup>. Based on the body surface area, the MTD corresponded to a dose of 960 mg/m<sup>2</sup>, which is much higher compared to the dose administered to humans (175 mg/m<sup>2</sup>) during HIPEC (19). This underlines one of the opportunities of HIPEC: the possibility to use higher doses, resulting in higher local concentrations which are maintained for a longer time in the abdominal cavity and which have a higher direct cytotoxic effect (20).

During HIPEC treatment, a sample of the perfusate was taken every 15 min in order to monitor the delivered PTX concentration. Statistical analysis showed no differences between the applied concentration of the different formulations ( $p=0.348$ ).

Monitoring the PTX plasma concentrations over a 90 min period (Fig. 5) showed similar concentrations for Taxol<sup>®</sup> and the nanosuspension during the perfusion period. However, when the cytotoxic agent was removed after



**Fig. 7** Tumor volume (mean  $\pm$  STD) (%) compared to day 0, measured by MRI 7 days and 14 days after HIPEC treatment with (■) no treatment, (■) Taxol<sup>®</sup> and (■) PTX/Plu F127 4/1 nanosuspension ( $n=6$ ).

HIPEC treatment (*i.e.* after 45 min) the PTX plasma concentrations of the nanosuspension increased, while PTX plasma levels after Taxol<sup>®</sup> treatment remained constant during the entire monitoring period (*i.e.* 90 min). The enhanced absorption of PTX was also reflected in the pharmacokinetic parameters after perfusion with a PTX nanosuspension: in comparison to Taxol<sup>®</sup>  $C_{\max}$  was significantly higher (124.7 ng/ml *vs.* 42.0 ng/ml,  $p=0.03$ ), and  $AUC_{t=90\text{ min}}$  was 1.5-fold higher but not significant different (95 % CI  $3.8 \pm 1.06 \mu\text{g}\cdot\text{min}/\text{ml}$  *vs.*  $2.5 \pm 0.212 \mu\text{g}\cdot\text{min}/\text{ml}$ ). Previous research already described that nanoparticles (>50 nm) can adhere to mucosa, thus prolonging the contact time of the drug and enhancing its absorption (21). Prolonged retention of PTX in the peritoneal cavity (in combination with enhanced PTX penetration) can offer a therapeutic advantage as tumor cells are exposed for a longer time to higher local drug levels. This approach overcomes one of the limitations of conventional intraperitoneal (IP) drug therapy where drugs are rapidly cleared from the peritoneal cavity (12). As the higher plasma levels using PTX/Plu F127 4/1 nanocrystals indicated more penetration through the peritoneum-plasma barrier, one can also assume a better penetration of the cytostatic in the tumor.

The effect of the PTX nanosuspension on tumor growth was evaluated *via* a tumor growth delay study, using Magnetic Resonance Imaging (MRI) as imaging technique to monitor tumor volume in a rat model. Although peritoneal carcinomatosis is characterized by a spread over the entire abdominal cavity, the rats were implanted with a single tumor nodule to simplify the tumor growth analysis (Fig. 6). At day 7 and 14 after HIPEC treatment with the PTX formulations, tumor growth was significantly different compared to the non-treated group ( $p=0.001$  and  $0.02$  for Taxol<sup>®</sup>, and  $0.003$  and  $0.010$  for the nanosuspension at day 7 and 14, respectively). No significant differences were observed between both PTX formulations ( $p=1.000$  and  $p=0.929$  at day 7 and day 14, respectively) (Fig. 7). The effect of the PTX/Plu F127 nanosuspension on the tumor volume was similar to Taxol<sup>®</sup>. At day 7 tumor volume was reduced for both PTX formulations, however the results were not significantly different from the tumor volume at day 0 ( $p=0.104$  and  $0.097$  for Taxol<sup>®</sup> and the nanosuspension, respectively). At day 14, the tumor volume had increased compared to the status at day 7 and was not significantly different from the initial tumor volume. Although little is known about the penetration of drugs in solid tumors, cytotoxic agents penetrate only a few millimeters into the tumor tissue, mostly *via* diffusion (22). Due to the limited penetration of anticancer drugs in solid tumors, IP chemotherapy is in the abdominal cavity only effective in micrometastases or tumors smaller than 5 mm in diameter (23). Hence, HIPEC could not completely eradicate the solid

tumor implanted in the rat model and 14 days after treatment the tumor volume had increased as a result of proliferation of the remaining tumor cells. However, in clinical practice cytoreductive surgery precedes HIPEC treatment, while HIPEC is used to remove the remaining tumor cells which are not visible and also to prevent the implantation of tumor cells at the resection site and on other abdominal and pelvic surfaces (2). Therefore, it is likely that in practice HIPEC using a PTX nanosuspension will remove all remaining microscopic tumors.

## CONCLUSION

A stable nanocrystalline paclitaxel formulation was developed *via* the wet milling technique using a high paclitaxel-to-stabilizer ratio. The cytotoxicity and antitumor efficacy in a rat model with peritoneal carcinomatosis of ovarian origin was similar to Taxol<sup>®</sup>. However, the advantage of using a non-cytotoxic excipient (Pluronic F127<sup>®</sup>) in the nanosuspension was reflected in the faster recovery of the rat after HIPEC treatment.

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