Paclitaxel nanoparticles for the potential treatment of brain tumors

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Abstract

Despite the advances in tumor therapy, patients with primary brain tumors and brain metastases have a very poor prognosis. Low responses to chemotherapy are mainly attributed to impermeability of the blood–brain barrier to cytotoxic agents. Paclitaxel has been shown to be active against gliomas and various brain metastases. However, its use in treatment of brain tumors is limited due to low blood–brain barrier permeability and serious side effects associated with administration of the paclitaxel solvent, Cremophor EL. Lack of paclitaxel brain uptake is thought to be associated with the p-glycoprotein (p-gp) efflux transporter. In this work, paclitaxel (PX) was entrapped in novel cetyl alcohol/polysorbate nanoparticles. Paclitaxel nanoparticles (PX NPs) were characterized by means of size, short-term stability, drug entrapment efficiency, and release profile. The PX NP cytotoxicity profile was monitored using two different cell lines, U-118 and HCT-15. Brain uptake of PX NPs was evaluated using an in situ rat brain perfusion model. The results suggest that entrapment of paclitaxel in nanoparticles significantly increases the drug brain uptake and its toxicity toward p-glycoprotein expressing tumor cells. It was hypothesized that PX NPs could mask paclitaxel characteristics and thus limit its binding to p-gp, which consequently would lead to higher brain and tumor cell uptake of the otherwise effluxed drug.

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1. Introduction

Paclitaxel (PX) is an anti-tumor agent with a unique mechanism of action [1,2]. It promotes the assembly of microtubules from tubule dimers and prevents them from depolarizing. This leads to loss of normal microtubule dynamics necessary for cell division and other vital processes, and consequently causes cell death. The ability of paclitaxel to stabilize the microtubules makes it an effective agent against various types of cancers. It has been demonstrated that paclitaxel is active against malignant gliomas and brain metastases [3,4]. However, low to undetectable
levels of the drug were found in the normal brain [5,6] as well as in tumor brain tissue [7]. Paclitaxel has been shown to be a substrate for the multidrug resistance 1 (MDR1) expressed protein, p-glycoprotein (p-gp) [4,5,8,9]. This efflux transporter has been reported to effectively limit brain penetration of various anticancer agents including paclitaxel [10,11]. A number of reports confirm increased brain penetration of paclitaxel after inhibition of efflux transporters at the BBB. Fellner et al. [4] have shown a 90% volume reduction of human glioblastoma tumors in mice after co-administration of paclitaxel with the p-gp inhibitor PSC833.

Paclitaxel has very low solubility in water and many pharmaceutically acceptable solvents [12]. The commercially available Taxol® formulation contains paclitaxel dissolved in a 50:50 v/v mixture of the surfactant Cremophor EL® (polyoxyethylated castor oil) and dehydrated ethanol. Cremophor EL has been shown to cause acute hypersensitivity reactions, one of the most severe side effects associated with administration of Taxol occurring in 2–4% of patients [1,12].

Many attempts have been made to formulate paclitaxel in a delivery system that does not require Cremophor for solubilization, including micelles, nanoparticles, liposomes, co-solvent systems, emulsions, and various conjugates [1,12]. Two of the more promising formulations include ABI-007 nanoparticles and poly-(L)-glutamic acid-paclitaxel conjugate (Xyotax™). ABI-007 is a formulation comprised of 150–200 nm paclitaxel nanoparticles prepared by high-pressure homogenization in the presence of albumin [13]. ABI-007 has shown promising results in both phase II and phase III clinical trials in head and neck cancer patients [14]. Xyotax™ is an ester-linked polymer–paclitaxel conjugate designed to increase aqueous solubility of paclitaxel and improve passive tumor targeting [15,16]. However, it is not expected that any of the above-mentioned formulations would efficiently deliver paclitaxel across the BBB. Therefore, the development of a new drug delivery system that not only eliminates Cremophor-related toxicity but also increases the efficacy of paclitaxel in the treatment of brain tumors is needed.

It has been recently reported that novel nanoparticles could be used as potential drug carriers across the blood–brain barrier [17,18]. Prototype nanoparticles were engineered from warm oil-in-water microemulsion precursors [19]. These nanoparticles are composed of the potentially biocompatible and biodegradable materials, such as cetyl alcohol/polsorbate 60 (Emulsifying Wax) and Brij 72 as the oil phase and Brij 78 and Tween 80® as the surfactants. Nanoparticles having diameters less than 100 nm were formed in a very reproducible, one-vessel procedure that did not require high-torque mechanical mixing. Emulsifying Wax (E. Wax) and Brij 72 nanoparticles had no effect in in vitro and in situ models on basic BBB parameters such as integrity, permeability, blood flow, and transport of choline [17]. Additionally, nanoparticles labeled with 3H-cetyl alcohol were shown to cross the BBB with a $K_{in}$ of $4.1 \pm 0.5 \times 10^{-3}$ ml/s/g. [18]. The goal of this work was to formulate paclitaxel in a nanoparticulate delivery system that does not contain Cremophor and that would be able to increase brain penetration of paclitaxel.

2. Materials and methods

2.1. Materials

$^3$H-paclitaxel (16.2 Ci/mmol) was a gift from the National Cancer Institute. $^{14}$C-paclitaxel (68 mCi/mmoll) was purchased from Moravek Biochemicals (Brea, CA) and $^{14}$C-sucrose (4.75 mCi/mmol) was obtained from Dupont-New England Nuclear (Boston, MA). Emulsifying wax (E. Wax), polyoxyethylene 20-sorbitan monooleate (Tween 80, polysorbate 80), and Float-A-Lyzers MWCO 3500 Da were purchased from Spectrum Chemicals (New Brunswick, NJ). Polyoxyl 20-stearyl ether (Brij 78) was obtained from Uniqema (Wilmington, DE). Paclitaxel (PX), Sephadex G-75, phosphate buffered saline (PBS), Cremophor EL, and anhydrous ethyl alcohol were purchased from Sigma (St. Louis, MO). Calf bovine serum, Dulbecco’s modified Eagle’s medium, and RPMI-1640 modified medium were obtained from American Type Culture Collection (Manassas, VA). Penicillin/streptomycin solution was purchased from Gibco Invitrogen (Carlsbad, CA). Materials were used as obtained. For all experiments, deionized water was filtered through 0.22-μm filters (Nalgene International, Rochester, NY).
2.2. Preparation of paclitaxel nanoparticles from microemulsion precursors

Microemulsion precursors were prepared as reported by Oyewumi and Mumper [19]. Briefly, 2 mg of E. Wax was weighed out into glass vials. Deionized, 0.22-μm filtered water was added and the mixture was heated to 50–55 °C under stirring conditions to melt the E. Wax. To the milky slurry of E. Wax in water, an aliquot of 100 mM Brij 78 was added to obtain a final volume of 1 ml and final surfactant concentration of 5 mM. Microemulsions formed spontaneously, and nanoparticles were then solidified by simple cooling of the warm microemulsions to room temperature under stirring conditions. To prepare PX NPs, various amounts of the drug were dissolved in the melted E. Wax and nanoparticles were solidified as described above. The final concentration of nanoparticles in all samples was 2 mg/ml.

2.3. Nanoparticle radiolabeling

To assess paclitaxel entrapment efficiency, nanoparticles were radiolabeled by entrapment of 14C-paclitaxel. Radioactive drug dissolved in ethyl acetate was pipetted into glass vials containing E. Wax and cold paclitaxel. Vials were left on a hot plate (50 °C) to allow complete evaporation of ethyl acetate. After evaporation of the solvent, nanoparticles were formulated as described in above. 14C-PX NPs had specific activity of 0.5 μCi/ml. For in vitro release and in situ transport studies, nanoparticles were radiolabeled with 3H-paclitaxel. All 3H-PX preparations were formulated with specific activities of 100 μCi/ml. 3H-NPs were radiolabeled by the entrapment of 3H-cetyl alcohol as described [18].

2.4. Characterization of paclitaxel nanoparticles (PX NPs)

Particle size was measured at 20 °C using a Coulter N4 Plus Sub-Micron Particle Sizer (Coulter, Miami, FL) at 90° light scattering for 90 s. Before size determination, nanoparticle suspensions were diluted with water to ensure light scattering intensities within the required range of the instrument (5×10^4 to 1×10^6 counts per second).

The size and morphology of PX NPs were observed using Philips Tecnai 12 Biotwin microscope at the University of Kentucky Medical Center Imaging Facility. A drop of nanosuspension was deposited on a copper mesh formvar carbon-coated grid and left to incubate for 1.5 min at room temperature. After removal of excess fluid, the samples were negatively stained with 2% uranyl acetate, and the grids were examined with the transmission electron microscope (TEM).

To evaluate PX NPs stability in biologically relevant media, nanoparticles were diluted 1:10 v/v with 150 mM sodium chloride (NaCl), water, or 10% fetal bovine serum (FBS) and incubated at 37 °C for 60 min. The stability of PX NPs was assessed based on the retention of particle size. Short-term stability of nanoparticle suspensions was determined after storage at 4 °C for 24 h. Before dilution for particle sizing, samples were left to equilibrate to room temperature. In addition, the chemical stability of paclitaxel in nanoparticles was tested by dissolving PX NPs in ethanol and quantifying paclitaxel and its degradation products by HPLC [20] and mass spectroscopy.

The entrapment efficiency of 14C-paclitaxel was determined using gel permeation chromatography (GPC). To obtain GPC elution profiles, 150 μl of radioactive nanoparticle suspension was eluted through Sephadex G-75 columns (150×70 mm) with 10 mM PBS (pH 7.4) as the mobile phase. Nanoparticles were detected by light scattering (CPS) and liquid scintillation counting (LSC). Additionally, a control sample of 250 μl of saturated 14C-paclitaxel solution was passed down the GPC column and the presence of radioactivity detected by LSC. The entrapment efficiency (E) was calculated based on the ratio of radioactivity eluted in the void volume (P1) and total radioactivity put on the column (Pt) from the relationship:

\[ P_1/P_t = E \]  

To further correlate paclitaxel distribution between nanoparticles and possible Brij 78 micelles, nanoparticles were formulated using increasing concentrations of surfactant (3.5–5 mM) and 14C-paclitaxel entrapment efficiency was determined as described above.

The radiolabeled compound release profile was assessed by a dialysis method. Radiolabeled nano-
particles (200 µl) were purified on the GPC column to remove unentrapped drug. A volume of 600 µl of purified radiolabeled nanoparticle suspension was pipetted into a dialysis membrane (MWCO 3500 Da) and dialyzed against 40 ml of 10 mM PBS (pH 7.4) with 0.1% Tween 80 for 24 h at 37 °C. At predetermined time points, 100 µl of sample was withdrawn and radioactivity was measured.

2.5. Cell culture

A human glioblastoma cell line (U-118) and human colorectal adenocarcinoma cell line (HCT-15) were obtained from ATCC. U-118 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf bovine serum and antibiotics (100 µg/ml of streptomycin and 100 unit/ml of penicillin) at 37 °C in a humidified incubator with 5% CO2. HCT-15 cells were incubated in modified RPMI-1640 supplemented as above. Cells were maintained in exponential growth phase by periodic subcultivation.

2.6. In vitro cytotoxicity

U-118 cells were seeded in 96-well plates at the density of 1×10^4 viable cells/well and incubated overnight to allow for cell attachment. Cells were incubated with PX NPs or Taxol at concentrations ranging from 5 to 100 nM for 48 and 72 h. To evaluate possible effect of formulation components on cell viability, cells were also incubated with blank nanoparticles and Taxol vehicle (Cremophor EL–ethanol mixture at 50:50 v/v) at the highest concentrations used for the study. To determine the effect of various concentrations of paclitaxel on cell viability the CellTiter 96® Aqueous Nonradioactive Cell Proliferation Assay (Promega) was used. Briefly, at predetermined time points, media containing various formulations was removed from the plates and a solution of MTS/PMS diluted in cell culture media was added. Cells were incubated for 4 h at 37 °C and absorbance was measured spectrophotometrically at 490 nm using an ELISA plate reader. Cell viability was calculated using Eq. (2):

\[
Cell \, viability \, (\%) = \left( \frac{Abs_s}{Abs_{ctrl}} \right) \times 100 \tag{2}
\]

where Abs_s is the absorbance of cells tested with various formulations and Abs_{ctrl} is the absorbance of control cells (incubated with cell culture media only). The IC_{50} value was defined as the drug concentration required to inhibit growth of the cells by 50% relative to controls. HCT-15 cells were incubated with paclitaxel formulations at concentrations ranging from 25 to 1000 nM.

2.7. Brain uptake studies

The uptake of ^3H-nanoparticles, ^3H-paclitaxel, and ^3H-PX NPs into brain was assessed using the in situ rat brain perfusion model [21] with modifications [22–24]. Briefly perfusions of 45 s were used to determine initial brain distribution volumes. A subsequent set of experiments utilized similar brain “loading” by 45 s perfusion, followed by a 30 s buffered physiologic saline wash. Washes were utilized to determine the amount of rapid brain efflux (i.e., as measured by the decrease in brain distribution volume). All studies were approved by Texas Tech University HSC Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. For brain uptake studies, radiolabeled nanoparticles were prepared on the day preceding the animal experiment. Vials were sealed and shipped at 4 °C to Texas Tech University (Amarillo, TX) where transport experiments were performed.

2.8. In situ rat brain perfusion

Male Fischer-344 rats (220–330 g; Charles River Laboratories, Kingston, NY) were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal). A PE-60 catheter filled with heparinized saline (100 units/ml) was placed into the left common carotid artery after ligation of left external carotid, occipital and common carotid arteries. Common carotid artery ligation was accomplished caudal to the catheter implantation site. The pterygopalatine artery was left open during the experiments [22]. Rat rectal temperature was monitored and maintained at 37 °C by a heating pad connected to a feedback device (YSI Indicating Controller, Yellow Springs, OH). The left common carotid artery was connected to a syringe containing buffered physiologic perfusion fluid containing [in mM]: NaCl 128, NaPO₃ 2.4, NaHCO₃.
29.0, KCl 4.2, CaCl 1.5, MgCl2 0.9, and d-glucose 9) with 1 μCi/ml 3H-nanoparticles (final nanoparticle concentration ~40 μg/ml) and 0.3 μCi/ml 14C-sucrose (to determine vascular volume). Perfusion fluid was filtered and warmed to 37 °C and gassed with 95% O2 and 5% CO2. The pH and osmolality of this solution were ~7.35 and 290 mOsm, respectively, immediately before perfusion. The perfusion fluid was infused into the left carotid artery with an infusion pump for periods of 45 s at 10 ml/min (Harvard Apparatus, South Natick, MA). This perfusion rate was selected to maintain a carotid artery pressure of ~120 mm Hg [21]. In subsequent experiments, after the 45 s labeled perfusion, a 30 s washout with tracer free fluid was used to allow efflux of 3H-nanoparticles or 3H-paclitaxel from brain parenchyma or brain microvascular endothelium [18,25]. Rats were decapitated and cerebral samples were obtained as previously described [24]. Briefly, the brain was removed from the skull, and the perfused cerebral hemisphere dissected on ice after removal of the arachnoid membrane and meningeal vessels. Brain regions were placed in scintillation vials and weighed. In addition, two 50-μl aliquots of the perfusion fluid were transferred to a scintillation vial and weighed. The brain and perfusion fluid samples were then digested overnight at 50 °C in 1 ml of 1 M piperidine. Ten milliliters of Fisher Chemical scintillation cocktail (Beckman, Fullerton, CA) was added to each vial and the tracer contents assessed by dual-label liquid scintillation counting. Dual-labeled scintillation counting of brain and perfusate samples were accomplished with correction for quench, background, and efficiency.

2.9. Kinetic analysis

Concentrations of 3H-nanoparticles, 3H-paclitaxel, or 3H-PX NP tracer in brain (Cbr) and perfusion fluid (Cpf) were evaluated as dpm/g brain or dpm/ml perfusion fluid, respectively. These values are expressed as a brain distribution volume ratio (Vd) from the equation:

\[ V_d = \frac{C_{br}}{C_{pf}} \]  

Distribution volumes can be directly compared provided perfusion duration and or washout time frames are similar [26]. Vascular volume was accounted for in all experiments by subtraction of 14C-sucrose vascular volume, which was concurrently measured. Previous work has shown that current concentration of nanoparticles do not alter cerebral perfusion flow (F) or vascular volume during in situ perfusions [17].

2.10. Statistical analyses

Data presented are from frontal cerebral cortex unless otherwise specified. 3H-nanoparticles, 3H-paclitaxel, or 3H-PX NP brain distribution volumes were compared with a paired t-test. For all data, errors are reported as the standard error of the mean unless otherwise indicated. (GraphPad Prism Version 3.00 for Windows, GraphPad Software, San Diego, CA).

3. Results

3.1. Preparation and characterization of paclitaxel nanoparticles

The potential use of two novel nanoparticle formulations as drug carriers across the blood–brain barrier has been recently reported [17,18,27]. Components of the system described herein include emulsifying wax (cetyl alcohol/polysorbate 60 in a 4:1 w/w ratio) used as the oil phase, water, and Brij 78 as the surfactant. Using oil-in-water microemulsion templates nanoparticle formulations containing up to 150 μg/ml of paclitaxel were successfully prepared. At paclitaxel concentrations higher than 150 μg/ml during microemulsion, formation turbidity was noted as well as the presence of a white precipitate after cooling the microemulsions to room temperature to form solid nanoparticles. Formation of PX NPs was confirmed by laser light scattering. It was determined that the incorporation of paclitaxel in nanoparticles had no effect on particle size and at all drug concentrations tested nanoparticles had sizes below 100 nm. The size and morphology of PX NPs were also confirmed by TEM (Fig. 1). To predict the potential stability of PX NPs in biological conditions, nanoparticles were incubated in various biologically relevant media at 37 °C. Under all conditions tested, no change in particle sizes were noted, indicating the potential stability of PX NPs in vivo. Similar results were obtained during short-term stability studies after incubation of PX NPs.
for 24 h at 4 °C. Paclitaxel chemical stability studies demonstrated that no degradation products were formed and approximately 50% of paclitaxel epimerized to 7-epitaxol during nanoparticle formulation (data not shown). However, epimerization could be avoided by formulating PX NPs in slightly acidic conditions, e.g., 5 mM acetate buffer (pH 4.5).

The entrapment efficiency of paclitaxel was determined by comparing the activity of 14C-PX NPs before and after separation on a gel permeation chromatography column. The data is shown in Table 1. It was observed that the experimental loading was linearly correlated to theoretical loading, suggesting that paclitaxel entrapment/adsorption may be dependent on the equilibrium between nanoparticles and possible Brij 78 micelles. To further determine influence of possible surfactant micelles on paclitaxel entrapment efficiency, PX NPs were engineered with increasing concentrations of Brij 78. However, as shown in Table 1, paclitaxel entrapment efficiency was not dependent on concentration of surfactant used and for all Brij 78 concentrations tested paclitaxel entrapment efficiency was approximately 50%. Additionally, for all paclitaxel concentrations tested, approximately 50% of the drug was found to be entrapped within the nanoparticles.

To evaluate the characteristics of paclitaxel release from nanoparticles, GPC purified PX NPs were dialyzed against 10 mM PBS (pH 7.4) with 0.1% NaCl. Paclitaxel entrapment efficiency was assessed using gel permeation chromatography (GPC) elution profiles. 14C-paclitaxel nanoparticles and free drug were detected using liquid scintillation counting. Data are presented as the mean ± S.D. (n=3–4).

### Table 1

<table>
<thead>
<tr>
<th>Theoretical loading (mg/ml)</th>
<th>Brij 78 (mM)</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>5.0</td>
<td>48.8±3.4</td>
</tr>
<tr>
<td>0.1</td>
<td>5.0</td>
<td>49.1±1.4</td>
</tr>
<tr>
<td>0.15</td>
<td>5.0</td>
<td>46.9±5.5</td>
</tr>
<tr>
<td>0.15</td>
<td>4.5</td>
<td>49.9±2.2</td>
</tr>
<tr>
<td>0.15</td>
<td>4.0</td>
<td>47.0±5.0</td>
</tr>
<tr>
<td>0.15</td>
<td>3.5</td>
<td>39.9±4.6</td>
</tr>
</tbody>
</table>

Paclitaxel entrapment efficiency was assessed using gel permeation chromatography (GPC) elution profiles. 14C-paclitaxel nanoparticles and free drug were detected using liquid scintillation counting. Data are presented as the mean ± S.D. (n=3–4).
Tween 80 at 37 °C. Due to very low aqueous solubility of paclitaxel, care was taken during all experiments to ensure sink conditions. Control samples included dialysis of paclitaxel suspension and physical mixture of blank nanoparticles with paclitaxel. As seen in Fig. 2, all paclitaxel was released from nanoparticles within 10 h. In addition, it was determined that there was no release of paclitaxel from PX NPs when the formulations were stored at 4 °C for 24 h (shipping conditions).

3.2. Cell culture studies

A human glioblastoma cell line (U-118) was chosen for this study because it is known that this cell line dose not express p-glycoprotein [4]. Therefore, cytotoxicity of paclitaxel entrapped in nanoparticles could be compared to that of the drug in Cremophor EL–ethanol. To ensure that the cytotoxicity was caused by the drug itself and not by formulation components, cells were incubated with blank nanoparticles and Cremophor EL–ethanol mixture at the highest concentrations used for the study. For all control samples, no decrease in cell viability was noticed, indicating lack of cytotoxicity of blank nanoparticles and Cremophor EL–ethanol mixture on U-118 cells at the conditions tested. The calculated IC₅₀ values for both paclitaxel formulations, as reported in Table 2, were not statistically different.

Paclitaxel-resistant HCT-15 cells were incubated with PX NPs and Taxol for 48 and 72 h. Concentrations of paclitaxel in Taxol formulations ranged from 25 to 1000 nM. In this range, no cytotoxicity on colorectal carcinoma cells was noted (Fig. 3). Concentrations of the paclitaxel in nanoparticle formulations ranged from 25 to 750 nM. The viability of HCT-15 cells was markedly decreased in presence of paclitaxel nanoparticles when compared to free paclitaxel. Calculated IC₅₀ values were 360±53 and 377±92 nM for PX NPs at 48 and 72 h, respectively. Nanoparticle doses of 2.3 μg/10⁴ cells (corresponding to 1000 nM of paclitaxel in PX NPs) resulted in a decrease in HCT-15 cell viability caused by the presence of blank nanoparticles. At this dose of blank nanoparticles, cell viability was equal to 68.6±13.9% and 64.6±10.5% for incubation times of 48 and 72 h, respectively.

3.3. In situ brain uptake of paclitaxel

To determine if nanoparticles had significant brain efflux, (1) brain distribution volumes of ³H-nanoparticles at 45 s perfusion time frames and (2)

![Fig. 2. Release profile of ³H-paclitaxel from nanoparticles. Purified nanoparticles (0.15 mg/ml of paclitaxel) were dialyzed against 10 mM PBS (pH 7.4) with 0.1% Tween 80 at 37 °C for 24 h. Data represents mean±S.D. (n=3).](image)

![Fig. 3. Human colorectal adenocarcinoma cell line viability in presence of PX NPs and Taxol, after incubation for 48 and 72 h. Data are presented as the mean±S.E.M. (n=12).](image)

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>IC₅₀ (nM) PX NPs</th>
<th>IC₅₀ (nM) Taxol</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>22.1±9.8</td>
<td>21.1±8.0</td>
</tr>
<tr>
<td>72</td>
<td>8.7±0.4</td>
<td>11.5±4.1</td>
</tr>
</tbody>
</table>

IC₅₀ values were determined after incubation of PX NPs and Taxol at various concentrations with U-118 cell line for 48 and 72 h. Data are presented as the mean±S.D. (mean of four independent experiments, n=3 for each experiment).
distribution after a 45 s perfusion with an immediate subsequent wash with tracer and nanoparticle free perfusate for 30 s were investigated. \(^{3}\)H-nanoparticles initial 45 s distribution volumes determined (0.195±0.02 ml/g) were calculated as described (Fig. 4a). The calculated distribution volume with a 30 s washout (0.178±0.004 ml/g) demonstrated no significant change from baseline (\(p>0.05\)), suggesting little to no efflux of loaded \(^{3}\)H-nanoparticles from brain to plasma in 30 s. Similar experiments were then carried out with \(^{3}\)H-paclitaxel in Taxol and \(^{3}\)H-paclitaxel entrapped in nanoparticles. Confirmation of \(^{3}\)H-paclitaxel efflux was evidenced by demonstrated significant (\(p<0.05\)) decrease in brain distribution volume from baseline (0.116±0.33 ml/g) after the 30 s washout (0.034±0.007 ml/g) (Fig. 4b). Of major significance, when \(^{3}\)H-paclitaxel was loaded into the nanoparticles, initial distribution volumes (0.096±0.01 ml/g) were not significantly different (\(p>0.05\)) from those after the washout (0.074±0.008 ml/g). The discrepancy in brain distribution volumes between \(^{3}\)H-NPs (Fig. 4a) and \(^{3}\)H-PX NPs (Fig. 4b) is due to the fact that only approximately 50% of paclitaxel was entrapped in nanoparticles. Therefore, in the perfusate, 50% of the drug was found within nanoparticles and 50% was in the form of a free paclitaxel.

4. Discussion

Patients with primary brain tumors as well as brain metastases have a very poor prognosis [28–30]. Once the brain metastases have developed, median survival is approximately 4–5 months [31]. The primary treatment for brain tumors includes whole brain irradiation, surgery, and stereotactic radiosurgery [32,33]. Chemotherapy has a poor outcome due to the low permeability of most anticancer agents through the blood–brain barrier. However, there is number of reports suggesting that the integrity of BBB in tumor brain is compromised by up-regulation of angiogenic processes in tumors leading to the formation of new blood vessels. These new vessels are leaky and thus allow for increased brain penetration of molecules otherwise excluded from the brain [32,34]. In this case, the disruption of the BBB is localized, leading to distribution of drugs in tumor core but not in the growing margins of the tumor tissue, where drug levels were very low to undetectable [35]. Gallo et al. [5] have demonstrated increased brain levels of paclitaxel in brain tumor over normal brain. Of great interest is the fact that these studies have also shown statistically significant increase in tumor brain concentration of paclitaxel in p-gp knockout mice over wild type. This further indicates that even in the presence of leaky brain vasculature, p-gp efflux has a dominant role in limiting paclitaxel brain uptake.

It was recently reported that E. Wax nanoparticles had significant brain uptake when compared to sucrose. Thus, these nanoparticles were suggested to potentially serve as drug carriers for various drug
molecules to the brain [18,27]. In the present studies, paclitaxel, a drug normally excluded from the brain, was entrapped in nanoparticles. It was hypothesized that by masking paclitaxel characteristics, nanoparticles will be able to enhance paclitaxel delivery to the brain for the potential brain tumor therapy.

Paclitaxel was entrapped in E. Wax nanoparticles with an entrapment efficiency of approximately 50%. Considering the low aqueous solubility of paclitaxel and the fact that increasing surfactant concentration did not effect paclitaxel entrapment, it is thought that unentrapped drug was adsorbed to the surface of the nanoparticles. The nanoparticle matrix (cetyl alcohol/ polysorbate 60) has amphiphilic properties and thus may not create a suitable environment for paclitaxel. The relatively fast release of paclitaxel may indicate that paclitaxel is found within nanoparticles in the form of a molecular dispersion rather than as crystallized drug. However, additional studies on the physical state of paclitaxel in the nanoparticles and its effect on the release rate and release mechanism need to be performed.

Two different cell lines were chosen for the in vitro cytotoxicity studies; a human glioblastoma cell line, which is sensitive to paclitaxel and a paclitaxel-resistant human colorectal adenocarcinoma. Results of the cytotoxicity studies on U-118 cell line confirm that during the nanoparticle engineering process paclitaxel remained stable and cytotoxic. The pattern of the cell viability in the presence of PX NPs and Taxol were similar, suggesting that paclitaxel entrapped in nanoparticles is equally potent toward tested tumor cell line as commercially available Taxol formulation. The IC50 values determined in these studies were consistent with values previously reported [4].

Human colorectal adenocarcinoma cell line (HCT-15) is one of the 61 cell lines chosen by The National Cancer Institute for screening new anticancer drugs. HCT-15 cells are well characterized and are known to intrinsically express the p-glycoprotein-mediated multidrug resistance [36,37]. HCT-15 cell viability significantly differs in presence of both paclitaxel formulations. IC50 values for PX NPs were lower than for Taxol, which may indicate lack of paclitaxel interaction with p-gp when the drug is entrapped in nanoparticles. The fact that there were no differences in IC50 values for PX NPs at different time points seems to confirm that only paclitaxel entrapped in nanoparticles is taken up by the cells. It is expected that PX NPs, which are taken up by the cells, release the drug within the cell, whereas remaining PX NPs release drug into cell culture medium. Once paclitaxel is released from nanoparticles (about 10 h as demonstrated by the in vitro release studies), longer incubation times should not influence cell viability because free or released drug is actively effluxed by p-gp.

There are reports suggesting that surfactants may alter the function of efflux transporters including p-gp [38]. Thus, Brij 78 may alter p-gp function; however, present studies with the HCT-15 cells suggest that it is unlikely because (1) total concentration of Brij 78 is half of that of Cremophor in Taxol (the highest concentrations used for the study were 56 and 24 µg/ml for Cremophor and Brij78, respectively), (2) although there may be some free Brij 78 surfactant in the PX NPs formulation, most of the surfactant is likely on the nanoparticle surface. Moreover, Brij 78 in the nanoparticle formulation is hypothesized to have different physical characteristics than free Brij 78 [18], and (3) there is no difference in IC50 values for PX NPs at different time points. Cremophor EL has been shown to be one of the most potent surfactants in reversing multidrug resistance among surfactants tested by Woodcock [38]. However, during these present experiments with HCT-15 cells, even the highest concentration of Cremophor failed to increase paclitaxel cell uptake to an extent where it had any effect on cytotoxicity. With nanoparticles made with Brij 78, not only there is a lower total concentration of Brij 78 in comparison to Cremophor, but also a significant amount of Brij 78 is thought to be embedded in the nanoparticle surface due to the formation of the nanoparticles from the microemulsions. Embedding of Brij 78 in the nanoparticles would lead to an even further decrease in concentration of the free Brij 78 [18]. Additionally, Lo [39] tested the multidrug resistance modulating effect of various surfactants including Brij 30, and concluded from these studies that both hydrophobic and hydrophilic moieties are necessary for the surfactant to invoke changes in the fluidity of the cell membrane, which result in inhibition of the activity of membrane proteins, such as p-gp. Thus, it could be hypothesized that immobilization of the hydrophobic chains of Brij 78 in nanoparticles would lead to a decrease in possible Brij 78 effect on p-
pg. Finally, if Brij 78 inhibited p-gp efflux of paclitaxel from the colorectal adenocarcinoma cells, a time-dependent decrease in cell viability would be expected as observed in the case of U-118 cell line. In total, these observations suggest that Brij 78 in these studies did not affect p-gp.

The feasibility of overcoming drug efflux at the BBB and subsequent higher brain uptake of paclitaxel was evaluated using in situ rat brain perfusion technique. During 45 s loading experiments, PX NPs and Taxol had similar brain uptake distribution profiles. This is attributed to minimal experimental time frames (e.g., not sufficient time for p-gp efflux). However, when the 45 s perfusion was followed by a 30 s washout with tracer-free saline, a statistically significant increase in brain distribution was observed for paclitaxel entrapped in nanoparticles when compared to the Taxol formulation. To ensure that brain uptake of paclitaxel in nanoparticles is not attributed to endothelium disruption, BBB integrity was monitored during all experiments with 14C-sucrose. The results agree with previously published data for blank nanoparticles [17] and indicated that there was no BBB opening in the presence of PX NPs.

The in situ data suggest that paclitaxel brain uptake was significantly increased by the use of nanoparticulate delivery system. Possible mechanisms include (1) the limited access of drug to p-gp by nanoparticle entrapment, (2) modulation of BBB p-gp by the surfactant, and (3) opening of the BBB in presence of nanoparticles. Opening of the BBB would be most concerning. However, this is unlikely, given that no increase in sucrose flux was measured during brain uptake experiments. Additionally, it was confirmed in previous in vivo and in vitro studies that E. Wax nanoparticles did not have any effect on baseline BBB parameters, such as integrity, permeability, blood flow, and active transport of choline [17].

References


