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Comparison of anti-tumor efficacy of paclitaxel delivered in nano- and microparticles

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ABSTRACT

This research compares the anti-tumor efficacy of paclitaxel delivered intratumorally in PLGA nanoparticles, microparticles, or the commercial Paclitaxel Injection®. The hypothesis of the research is that larger PLGA microparticles adhere to mucus on the cell surface, release paclitaxel locally, and enhance cellular association of paclitaxel. PLGA-paclitaxel particles of mean diameters 315 nm, 1 μm, and 10 μm were prepared and their drug content, *in vitro* release, and cellular association of paclitaxel into 4T1 cells quantified. These particles were injected intratumorally into tumor xenografts, and the tumor volumes monitored over 13 days. Mean tumor volumes of the groups that received placebo and the 315 nm nanoparticles increased 2 and 1.5 times, respectively. Tumor growth was arrested in groups that received 1 μm and 10 μm microparticles. Additional cell culture studies were performed to test the hypothesis. The size-dependent increase in cellular concentration of paclitaxel was independent of duration of incubation of PLGA particles with 4T1 cells, and was enhanced 1.5 times by coating the particles or 4T1 cells with mucin. These particles were not internalized by clathrin-mediated endocytosis or macropinocytosis. In conclusion, PLGA microparticles sustained drug release, increased cellular concentration, and enhanced anti-tumor efficacy of paclitaxel compared to nanoparticles and Paclitaxel Injection®.

1. Introduction

In 2009, it is expected that breast cancer will account for 27% of all the newly diagnosed cancer cases in women (Jemal et al., 2009). Paclitaxel is approved to treat breast and ovarian cancer and has also demonstrated anti-cancer activity against non-small cell lung cancer as well as Kaposi's sarcoma (Tulpule et al., 2002). Paclitaxel stabilizes microtubules against depolymerization, resulting in altered cellular functions during mitosis leading to apoptosis (Spencer and Faulds, 1994).

It is well documented that the low aqueous solubility of paclitaxel creates formulation challenges. The first commercially available intravenous formulation of paclitaxel is prepared by dissolving the drug in a 50:50 mixture of Cremophor® EL (polyethoxylated castor oil) and dehydrated alcohol (Panchagnula, 1998). However, Cremophor® EL was reported to cause anaphylactic hypersensitivity reactions, hyperlipidaemia, neurotoxicity, and alteration of paclitaxel's pharmacokinetics (Gelderblom et al., 2001). In 2005, the FDA approved Abraxane®, a solvent-free, albumin-bound paclitaxel, for the treatment of metastatic breast cancer. Although Abraxane® addressed the disadvantages of Cremophor, during phase III clinical trials, patients developed neutropenia and sensory neuropathy (Gradishar et al., 2005). Alternate delivery systems for paclitaxel have been, and continue to be investigated including emulsions, micelles, liposomes, cyclodextrins, implants, pastes and prodrugs (Singla et al., 2002).

Intratumoral chemotherapy provides sustained, localized, and elevated drug concentrations with low systemic toxicity and may prevent post-operative metastasis (Goldberg et al., 2002). An ideal formulation for intratumoral administration must have the following characteristics: (1) be injectable into the tumor mass, (2) provide sustained, predictable drug release, (3) be stable in the tumor milieu, (4) and be non-toxic. Therefore, injecting particulate delivery systems directly into tumors, or the resected tumor space could enhance the anti-tumor efficacy compared to systemic intravenous injection. Poly D, L-(lactide-co-glycolide) [PLGA] has been widely used as a polymeric vehicle for controlled release of hydrophilic and hydrophobic drugs. The formulation parameters affecting the particle size, drug loading, and *in vitro* release

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of hydrophobic drugs from the PLGA particles have been reviewed (Wischke and Schwendeman, 2008). While the effect of size of the PLGA particles on the *in vitro* drug release of encapsulated drugs has been well investigated (Klose et al., 2008; Budhian et al., 2008), the impact of PLGA particle size on the anti-tumor efficacy of the encapsulated drug following intratumoral administration has not been compared.

It has been previously shown that when delivered in nanoparticles, paclitaxel was more cytotoxic than the commercially available formulation (Chen et al., 2001; Fonseca et al., 2002). In an intratumoral chemotherapy study using emulsified wax/Brij® 78 nanoparticles containing paclitaxel, a 50% reduction in tumor volume after 19 days was reported compared to the same dose of paclitaxel in solution (Koziara et al., 2006). Lapidus et al. (2004) reported a 50% reduction in tumor volume after intratumoral administration of paclitaxel in polilactofate (Paclimer®) microparticles. More recently, Xie et al. (2007) reported that intratumoral injection of paclitaxel-loaded PLGA microparticles (42.72 μm) reduced tumor weight by 59.9% over a 21-day treatment period, compared to paclitaxel injection. These authors also reported that Vascular Endothelial Growth Factor (VEGF) expression was significantly lower in PLGA microparticle-treated tumors suggesting that this delivery system may enhance the anti-angiogenic activity of paclitaxel. Another study reported that when delivered in microparticles, paclitaxel exhibited greater anti-tumor efficacy than the commercial product (Harper et al., 1999; Lapidus et al., 2004). When co-injected with Lewis Lung Carcinoma Cells into the subcutaneous tissue, paclitaxel-containing PLGA microparticles of size range 1–5 μm significantly inhibited tumor growth and cell proliferation compared to placebo-PLGA microparticles (Azouz et al., 2008). *In vitro* cell culture studies of paclitaxel-loaded PLGA microparticles of the size range 0.5–5 μm demonstrated a dose-dependent cytotoxicity on human uterine cancer cells, with IC_{50} values similar to that of Taxol® (Hamoudeh et al., 2008).

Based on this literature, it can be concluded that greater cellular accumulation of paclitaxel is achieved when delivered in either nanoparticles or microparticles compared to solution. However, a comparison of the *in vitro* cellular association, cytotoxicity, and anti-tumor efficacy of paclitaxel-containing PLGA nanoparticles and microparticles has not been previously reported. We reported that a greater cellular association of paclitaxel into 4T1, Caco-2, and Cor-L23/R cells was achieved when delivered in microparticles compared to nanoparticles (De et al., 2005). It was hypothesized the larger microparticles adhere to mucus on the cell surface, enhancing the cellular association of paclitaxel. This hypothesis was supported by additional experiments using confocal microscopy in which it was demonstrated that microparticles adhere to the surface of 4T1 cells (De et al., 2005). Therefore, the specific aims of this study were to (1) compare the efficacy of paclitaxel delivered locally in nanoparticles, microparticles and the commercially available formulation administered intravenously and intratumorally in a mouse model using the 4T1 murine mammary adenocarcinoma cell line; and, (2) to investigate the mechanism responsible for the increased cellular association of paclitaxel delivered in larger microparticles compared to nanoparticles. Placebo and paclitaxel-containing PLGA nanoparticles and microparticles were administered intratumorally into xenografts developed on balb/c mice and the tumor growth was monitored over 13 days. To investigate the mechanism underlying the hypothesis that the observed size-dependent increase in cellular association of paclitaxel delivered in larger microparticles is due to the adhesion of microparticles to mucus, *in vitro* cellular association studies were performed after (a) different durations of incubation of the PLGA particles with the 4T1 cells, (b) use of metabolic inhibitors.

2. Materials and methods

2.1. Materials

Female *balb/c* mice, 6 weeks of age, were obtained from Charles River Laboratories, Wilmington, MA. Paclitaxel, poly-vinyl alcohol (PVA), mucin (Type II, from porcine stomach) potassium chloride, monobasic sodium phosphate, sodium chloride, sucrose, dibasic sodium phosphate, cytochalasin B, and ketamine/xylazine hydrochloride were purchased from Sigma–Aldrich, St. Louis, MO. PLGA 50:50 with inherent viscosity of 0.63 dL/g was purchased from Birmingham Polymers, Inc. Commercial Paclitaxel Injection® was purchased from NovaPlus, IVAX Laboratories, Inc.

2.2. Preparation of PLGA-paclitaxel nano- and microparticles

Nano- and microparticles were prepared using the conventional o/w emulsion-solvent evaporation technique (Bala et al., 2004). Briefly, 3–4 mg of paclitaxel and 90 mg of PLGA were dissolved in 3 mL of dichloromethane. This solution was then emulsified with 25 mL of a 1.5% (w/v) aqueous solution of PVA and stirred overnight allowing the solvent to evaporate. The resulting suspension was centrifuged at 33,000–40,000 rpm for 25 min at 4 °C. The supernatant was discarded and the pellet resuspended in water. This procedure was repeated twice. The sample was lyophilized and stored at 4 °C until used. Placebo-PLGA particles were prepared using an identical protocol.

Particles containing paclitaxel were prepared in three sizes approximately 315 nm, 1 μm, and 10 μm by varying the emulsification intensity during preparation with a probe sonicator (Misonix Inc.) or the omni-mixer (Sorvall). Each instrument was previously calibrated to prepare particles of the desired size based on energy of emulsification.

2.3. Characterization of delivery systems

The mean particle diameter of the nanoparticles and microparticles was determined using dynamic light scattering using an aqueous suspension of the particles (0.5 mg/mL). The morphology of the PLGA particles was observed using scanning electron microscopy. In addition, the mean particle size of the microparticles was determined from the scanning electron photomicrographs. The images of both nano- and microparticles were recorded after coating them with chromium at 95 mA.

The drug content of the particles was determined in triplicate after extraction and paclitaxel concentration quantified using a HPLC assay described below. A known amount (~2 mg) of the particles were dissolved in 1 mL of dichloromethane and 0.5 mL of acetonitrile, vortexed, and then agitated at 37 °C for 30 min to ensure complete dissolution of polymer. The solvent was evaporated by flushing with nitrogen (20 psi). Acetonitrile (5 mL) was added to the extract and agitated overnight in a reciprocal shaking bath at 37 °C and 50 rpm to dissolve paclitaxel. The drug content in the acetonitrile solution was analyzed by reverse-phase HPLC using a C-18 column (Curosil® PFP). A 10 μL sample was injected and a mobile phase consisting of 60:40 (v/v) of acetonitrile and water used at a flow rate of 1 mL/min. A calibration curve was used to determine the drug content. The loading efficiency was determined from the drug content, and is the ratio of the drug content to the theoretical amount of drug used in the preparation of the PLGA particles. It is expressed as a percentage.

2.4. In vitro drug release

An accurately known mass of nanoparticles and microparticles of paclitaxel containing the equivalent of approximately 30 μg of paclitaxel was suspended in 25 mL of dissolution medium

and agitated in a reciprocal shaking bath at 50 rpm and 37 °C. At predetermined time intervals, each dissolution sample was removed from the water bath, centrifuged at 33,000–40,000 rpm, and the supernatant collected. The pellet was resuspended in 25 mL of fresh dissolution medium, sonicated mildly in a water bath to disperse the aggregated particles, and the study continued. After the supernatant was lyophilized, the remaining drug in the residue was extracted in to 2 mL of acetonitrile and quantified using the HPLC quantitative assay procedure described previously.

2.5. In vitro cellular association of paclitaxel delivered in nanoparticles or microparticles

Murine, metastatic breast carcinoma (4T1) cells were used to compare the cellular concentration of paclitaxel following delivery in nanoparticles or microparticles. The cells were propagated in complete media and when confluent, the cells were seeded into 6-well plates at 250,000 cells/well and allowed to grow for four days. The cells were then treated with media containing paclitaxel in either solution, nanoparticles, or microparticle formulations. After 90 min of incubation, the media was discarded, the cells were washed thrice to remove non-adherent particles, scraped, and lyzed. The total cell protein was determined using a BCA® protein assay (Pierce) that detects proteins colorimetrically. The lyzed cell suspension was lyophilized and the paclitaxel was dissolved into 500 μL of acetonitrile. The concentration of paclitaxel associated with the cells was analyzed using the HPLC assay as described previously.

2.6. Time-dependence of cellular association of paclitaxel delivered in nanoparticles and microparticles

After growing cells in six-well plates at 250,000 cells/well for 4 days, they were incubated with a solution, nanoparticles, or microparticle formulation containing paclitaxel for <1 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, 180 min, 240 min, and 300 min. The protein content and paclitaxel concentration were then determined as described previously.

2.7. Effect of mucus pre-treatment on the cellular association of PLGA nanoparticles and microparticles

A 1 mg/mL aqueous solution of porcine mucin was prepared, adjusted to pH = 6.0. The 4T1 cells were seeded into six-well plates at 250,000 cells/well. The two approaches adopted to study the effect of mucus on the cellular association of paclitaxel include coating either the 4T1 cells or the PLGA particles with mucin. The 4T1 cells were coated with mucin by incubating them in 1 mL of media containing 200 μL of mucin solution for 15 min at 37 °C and an atmosphere of 5% CO_2. A 1 mL suspension of PLGA nanoparticles or microparticles in the media was then added to each well. After incubating the cells for 90 min at 37 °C and 5% CO_2, they were washed thrice with ice-cold PBS, scraped, lyzed, and the paclitaxel concentration quantified by HPLC. As a control, identical experiments were performed with 4T1 cells that had not been exposed to mucin. To coat the PLGA particles with mucus, 1 mL of the suspension of PLGA particles was incubated with 250 μL of mucin for 15 min following which they were suspended in 4T1 cell media. The 4T1 cells were then incubated with the coated PLGA particles for 90 min at 37 °C and 5% CO_2. The cellular association of paclitaxel was then quantified after lyzing the cells. PLGA particles that had not been coated with mucin were used as the controls.

2.8. Clathrin-coated pit inhibition using Hypertonic Bicarbonate Ringer's Solution (HBRS)

HBRS was prepared by dissolving each of the following substances in double distilled water: calcium chloride (1.8 mM), potassium chloride (5.6 mM), magnesium sulfate (0.8 mM), disodium hydrogen phosphate (0.8 mM), sodium chloride (116 mM), sodium hydrogen carbonate (25 mM), HEPES (15 mM), D-glucose (5.5 mM), and sucrose (0.45 mM). Prior to incubation in media containing the PLGA particles, the cells were washed with assay buffer and incubated with 2 mL of HBRS for 0.5 h. The cellular association of paclitaxel was determined. Control experiments were performed using assay buffer alone.

2.9. Inhibition of macropinocytosis using cytochalasin B

A stock solution of 1 mg/mL of cytochalasin B was prepared using DMSO. After attaining confluence, the 4T1 cells were pretreated with 2 mL of media containing 30 μM cytochalasin B for 0.5 h. Untreated cells served as the controls. After 0.5 h of pretreatment, the cells were washed and incubated with media in which PLGA nanoparticles and microparticles were suspended, each containing the equivalent of 25 μg of paclitaxel/well. The cellular association of paclitaxel was quantified using HPLC.

2.10. Comparison of anti-tumor efficacy of the delivery systems

The *in vivo* anti-tumor efficacy of paclitaxel was determined in balb/c mice. All experiments adhered to the Principles of Laboratory Animal Care and have been approved by Institutional Animal Care and Use Committee at the University of Nebraska. To initiate tumor growth, 4T1 cells were first suspended in phosphate buffered saline and 100 μL of this suspension, equivalent to 10^5 cells, injected into the right rear flank of *balb/c* mice. Vernier calipers were used to measure tumor dimensions for approximately 20 days after injection and before initiating treatment. The length (a) and breadth (b) of the tumor were recorded and the tumor volume was calculated using the formula:

$$\text{Volume in mm}^3 = \frac{a + b^2}{2} \quad (1)$$

On the 24th day, when the tumors were in the exponential growth phase, the mice were randomly assigned to one of six groups receiving the following treatments: group 1 received placebo-PLGA particles, group 2 received paclitaxel-PLGA nanoparticles (315 nm), groups 3 and 4 received paclitaxel-PLGA particles of mean diameters 1 μm and 10 μm, respectively, and groups 5 and 6 received i.v. or intratumoral injections of Paclitaxel Injection®, respectively. Each mouse received the same dose of 24 mg/kg of paclitaxel. To administer the treatments, the mice were anesthetized by an intraperitonial injection of a solution of 80 μL of ketamine/xylazine solution containing 80 mg/mL and 6 mg/mL of ketamine and xylazine, respectively. The nano- and microparticles were suspended in 50 μL of phosphate buffered saline and administered intratumorally. The commercially available Paclitaxel Injection® was injected into the tumors (group 5) and intravenously through the tail vein. Throughout the treatment phase of the study, the mean diameters of the tumors were recorded approximately every 2 days and tumor volumes calculated using Eq. (1). The study was continued until either the tumor had regressed completely or, until the mice exhibited visual signs of distress and discomfort. At this time, the mice were euthanized in a CO_2 chamber. The changes in tumor volumes were statistically compared by one-way ANOVA using SPSS statistical software. Pairwise comparisons were carried out by Tukey's test. The body weight of mice before, and after treatment was recorded and statistically compared.

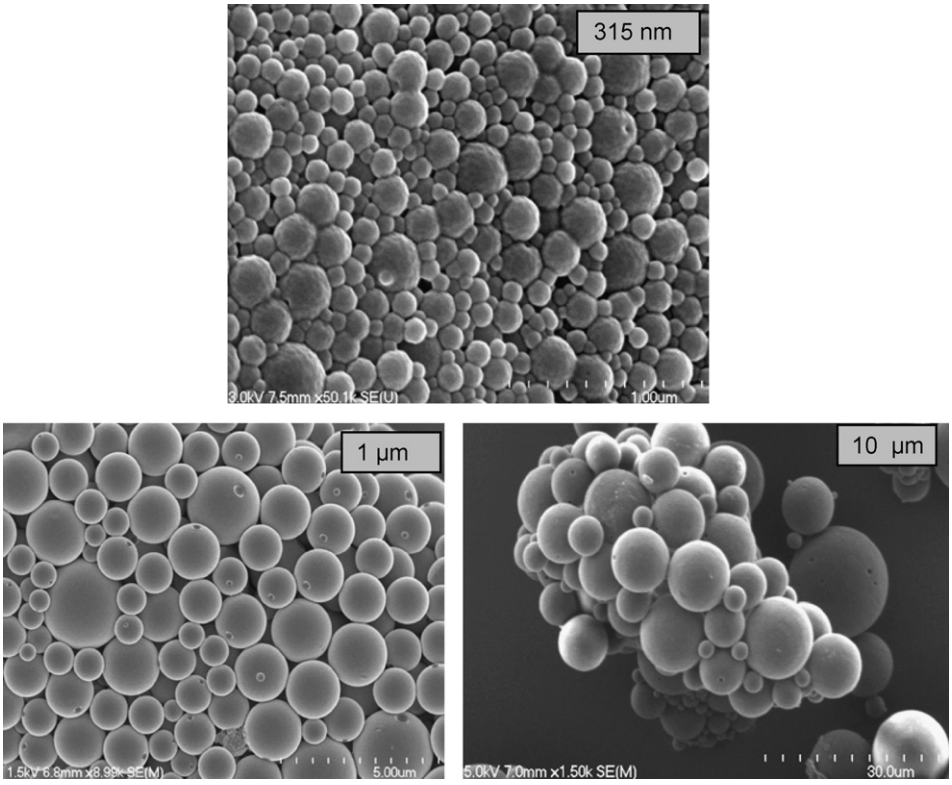

Fig. 1. Scanning electron photomicrographs of paclitaxel-loaded nanoparticles and microparticles.

3. Results and discussion

3.1. Particle size and in vitro drug release

The mean particle diameters of the PLGA particles were 315 nm, 1 μm, and 10 μm and the drug content in these nano- and microparticles was 3.72 ± 0.045%, 2.72 ± 0.016%, and 2.64 ± 0.057% (w/w), respectively. The average loading efficiency of paclitaxel into the PLGA nano- and microparticles was 95%. Representative scanning electron photomicrographs of the particles of varying sizes are shown in Fig. 1. Fig. 2 illustrates that, within 24 h, there was complete release of paclitaxel from the 315 nm nanoparticles, while the microparticles sustained drug release with 80% of the drug released over 24 days. Consistent with the literature, there was a significant burst effect with about 50% of the drug released from the nanoparticles in 6 h. The higher surface area to mass ratio of the nanoparticles contributes to their faster degradation, and therefore faster release of paclitaxel (Almond et al., 2003). However, the burst effect was significantly lower among the 1 μm and the 10 μm microparticles, with about 30% of the drug released in 6 h. The release profiles did not fit either zero- or first-order kinetics.

3.2. Cellular association of paclitaxel delivered in PLGA particles of various sizes

The concentration of paclitaxel associated with the 4T1 cells is shown in Fig. 3. There was no significant difference between the cellular concentrations of paclitaxel when delivered as 315 nm nanoparticles compared to the solution indicating that nanoparticles are no more efficient than a solution in elevating cellular drug levels. Most significantly, the cellular association of paclitaxel was 5 and 7 times greater when delivered in 1 μm and 10 μm microparticles, respectively, compared to nanoparticles again indicating that microparticles enhanced cellular concentration of the drug compared to either nanoparticles or a solution of the drug.

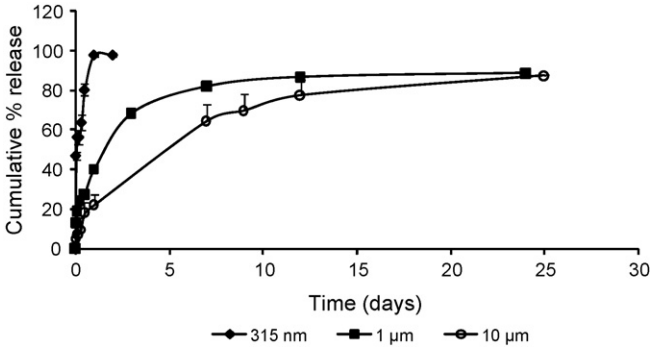

Fig. 2. Cumulative percent release versus time of paclitaxel from nanoparticles and microparticles.

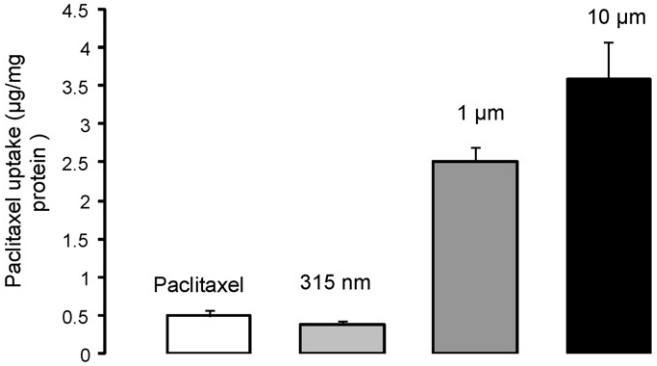

Fig. 3. Cellular concentration of paclitaxel associated with 4T1 cells.

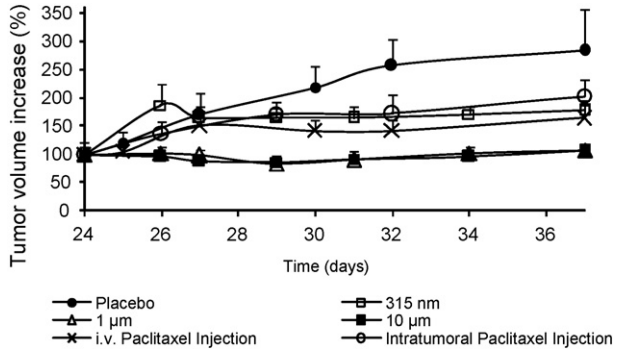

Fig. 4. Percentage increase in tumor volume of treatment groups compared to the placebo-treated group.

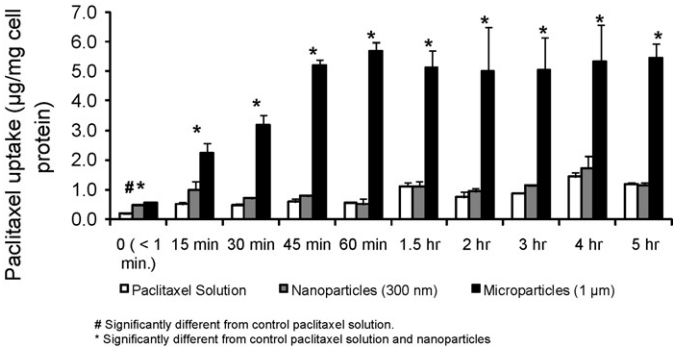

Fig. 6. Effect of incubation time on the cellular association of PLGA particles into 4T1 cells.

3.3. *In vivo anti-tumor efficacy study*

The relative changes in the tumor volume among the treatment groups are compared in Fig. 4. Further, the relative tumor volumes of all the groups on the 37th day, when the animals were euthanized, were statistically compared using one-way ANOVA with pairwise comparisons performed by Tukey's test.

The tumor volume of the placebo-treated mice increased 3-fold during the 13 days of treatment. By comparison, there was only a 1.5-fold increase in tumor volume observed in mice treated with 315 nm nanoparticles. Most significantly, tumor growth was totally suppressed in the groups that received paclitaxel in 1 μm and 10 μm microparticles compared to the placebo-treated group. As expected, no significant tumor regression was observed in the placebo-treated group confirming that PLGA does not have anti-tumor activity. When paclitaxel was administered either intravenously or intratumorally, the commercial product did not significantly regress tumor growth and therefore, is no more effective than the placebo-treated group. These observations were confirmed by the pairwise statistical comparison tests which indicated that, compared to the placebo-treated group, the relative tumor volume of the groups that received 1 μm and 10 μm microparticles were significantly different ($p < 0.05$), whereas the groups that received the commercial Paclitaxel Injection® did not demonstrate a statistically significant reduction in tumor volume.

A 11–22% increase in the mean body weight all the groups was recorded over the course of the study. However, there was no statistically significant difference in the body weights of the mice between the groups before treatment or at the time when euthanized (Fig. 5). Thus, the conclusion from this efficacy study is that microparticles were more efficient than the nanoparticles and the commercial product in suppressing tumor growth *in vivo* in the mouse model.

These results demonstrate that paclitaxel, when delivered in microparticles of sizes 1 μm and 10 μm, arrests tumor growth compared to nanoparticles. However, anti-tumor efficacy of paclitaxel was comparable when delivered the 1 μm and 10 μm microparticles, leading to the hypothesis that the cellular mechanism underlying the anti-tumor efficacy of larger microparticles of size range 1–10 μm is different compared to nanoparticles of size 300 nm. Therefore, subsequent experiments focused on investigating the mechanism of increase of cellular association of paclitaxel delivered in nanoparticles of size range 300–400 nm and microparticles of size range 1–1.5 μm.

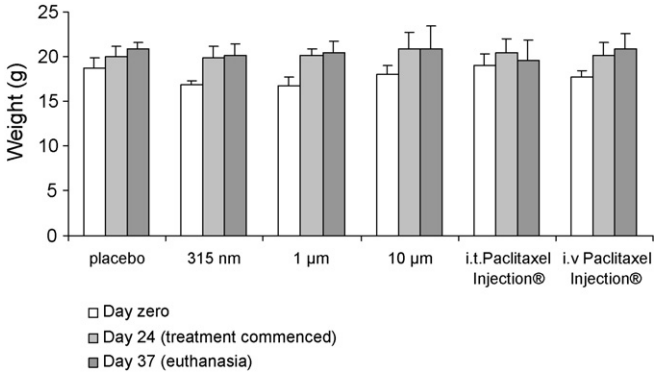

Fig. 5. Weight changes recorded in untreated and treated mice in each study group.

3.4. *Relationship between incubation time and the amount of paclitaxel associated with the cells after delivery in nano- or microparticles*

The cellular association of paclitaxel with 4T1 cells after delivery in PLGA nanoparticles of size 300 nm and microparticles of size 1 μm onto 4T1 cells was quantified at 10 time points from 0 to 5 h. Fig. 6 illustrates that, at all the incubation times tested, the cellular association of paclitaxel into 4T1 cells was 1.1–4.9-fold greater when delivered in the larger microparticles compared to smaller nanoparticles or solution. An important observation is that, when incubated for <1 min, the cellular association of paclitaxel was significantly greater when delivered in microparticles than nanoparticles.

3.5. *Effect of mucus pre-coat on the 4T1 cells or the PLGA nanoparticles and microparticles on the cellular association of paclitaxel*

This research aimed to establish if an increase in concentration of mucus on the surface of 4T1 cells will also enhance the adhesion and retention of nanoparticles and microparticles resulting in an increase in the cellular association of the drug. Similarly, it is hypothesized that pre-coating the particles with mucus will also enhance their binding onto the surface of the cells. Fig. 7 illustrates that, when the 4T1 cells were coated with mucus, the cellular association of paclitaxel from nanoparticles of size 296 nm increased 1.7-fold compared to the cellular association in uncoated 4T1 cells. Similarly, a 1.5-fold greater cellular association of paclitaxel was observed with nanoparticles coated with mucin compared to uncoated particles. On the other hand, uncoated PLGA microparticles of size 1.1 μm did not increase cellular association on mucus-coated 4T1 cells, whereas a 1.6-fold increase in cellular association was observed with mucus-coated microparticles (Fig. 8).

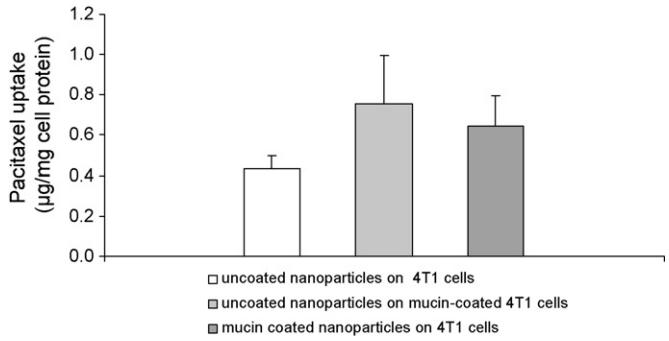

Fig. 7. Effect of mucus pre-treatment on PLGA nanoparticles and 4T1 cells on the cellular association of paclitaxel.

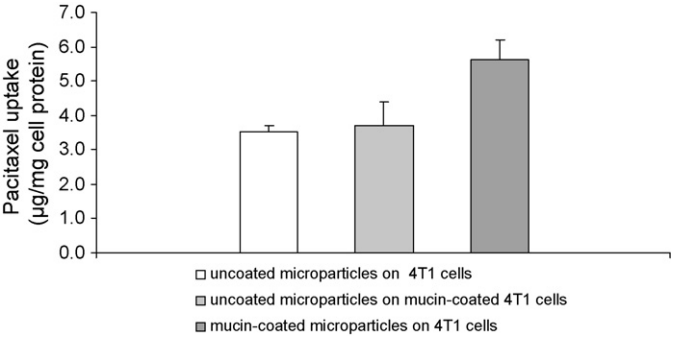

Fig. 8. Effect of mucus pre-treatment on PLGA microparticles and 4T1 cells on the cellular association of paclitaxel.

3.6. The role of endocytotic pathways on the cellular internalization of PLGA nano- and microparticles

As illustrated in Fig. 9, no significant difference in the cellular association of paclitaxel from PLGA particles was observed after pre-treatment with hypertonic BRS confirming that clathrin-mediated endocytosis was not responsible for the cellular association of the particles. Similarly, the size-dependent cellular association of paclitaxel from the nanoparticles and microparticles was sustained even after treatment with cytochalasin B suggesting that larger microparticles were not internalized into the cells (Fig. 10). Based on these experiments, it was concluded that the PLGA microparticles are retained on the cell surface without internalization.

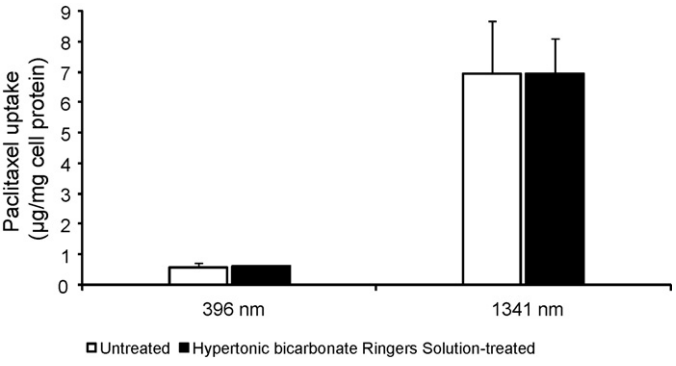

Fig. 9. Effect of hypertonic Bicarbonate Ringers Solution on the cellular association of paclitaxel delivered in PLGA particles.

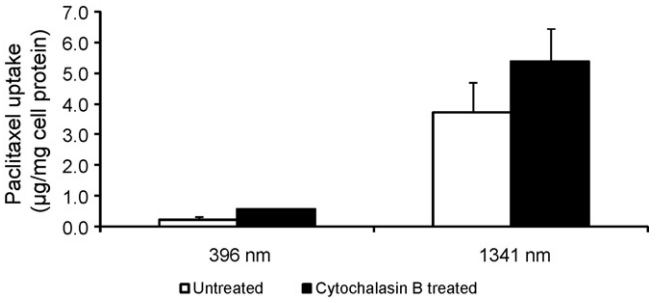

Fig. 10. Effect of cytochalasin B on the cellular association of paclitaxel delivered in PLGA particles.

4. Discussion

Because of the low aqueous solubility of paclitaxel, a differential dissolution study of the release of paclitaxel from PLGA particles was monitored in a medium consisting of 0.1% (w/v) aqueous Tween 80®. The solubility of paclitaxel in an aqueous solution of 0.1% (w/v) Tween 80® has previously been determined to be 5.32 ± 1.07 μg/mL (De, 2003). The release of paclitaxel from the microparticles was sustained for 14 days.

Although all the mice were injected with a cell suspension containing the equivalent of 10^5 4T1 cells, the tumor growth rate varied within, and between, groups. The average tumor volumes of the groups were significantly different even before treatment commenced and therefore, it was not possible to compare the absolute tumor volumes. The tumor volume 24 days after initiating treatment was taken as 100% and all subsequent tumor volume measurements were expressed as an increase or decrease from this baseline. The suppression of tumor growth in mice that received paclitaxel in 1 μm and 10 μm microparticles is consistent our previous findings that paclitaxel, when delivered in larger particulate formulations increased cellular drug concentrations and cytotoxicity (De et al., 2005).

Further, *in vitro* cell culture experiments demonstrated that the cellular association of paclitaxel into 4T1 cells delivered in PLGA nano- and microparticles is not affected by the duration of incubation of the PLGA particles with the tumor cells. An explanation for this phenomenon is that since cellular internalization of PLGA particles is negligible within 1 min, the higher paclitaxel concentration that resulted with microparticles was because of their adhesion to the cell surface increasing localized drug concentration. Except for the 1 min incubation period, the cellular association of paclitaxel from nanoparticles was never significantly greater than the control drug solution. Conversely, at all time intervals tested, the cellular association of paclitaxel was 1.1–4.9 times greater when delivered in microparticles compared to both nanoparticles and solution. These results support our hypothesis that PLGA microparticles may be retained on the surface of cell that increases the cellular association of paclitaxel.

Further, the following conclusions can be reached from the studies that quantified the cellular association of paclitaxel from PLGA particles upon pre-treatment of 4T1 cells or PLGA particles with porcine mucin: (1) increasing the mucus concentration on the cell surface enhanced the cellular association of paclitaxel from the nanoparticles but not the larger microparticles; (2) pre-coating both the PLGA nanoparticles and microparticles with mucin increased the cellular association of paclitaxel; and (3) these extent of adhesion and retention of PLGA particles by mucus on the cell surface is dependent on the particle size.

Pinocytosis is the predominant mode for the internalization of cellular cargo. Pinocytosis, or fluid phase endocytosis occurs through four mechanisms which include (a) clathrin-dependent endocytosis, (b) caveolin-mediated internalization, (c) macropinocytosis, and (d) clathrin and caveolin-independent pathways (Liu and Shapiro, 2003). Previous research demonstrated that the cellular association of paclitaxel delivered in nanoparticles or microparticles was non-specific and not mediated by the p-glycoprotein or breast cancer resistance protein (BCRP) efflux pumps (De et al., 2005). It has also been reported that caveolin-mediated endocytosis does not mediate internalization of particles into tumor epithelium (Conner and Schmid, 2003). While smaller particles may be internalized by clathrin-mediated pathways, macropinocytosis is the only mode of uptake of particles larger than 1 μm (Conner and Schmid, 2003). Clathrin-mediated endocytosis can be inhibited by hypertonic media such as hypertonic bicarbonate Ringer's solution, or intracellular potassium depletion, while macropinocytosis can be arrested by cytochalasin B (Qaddoumi et al., 2003). Cytochalasin is a fungal mycotoxin that inhibits the formation of actin filaments which are responsible for the internalization of PLGA microparticles of size >1 μm. A 30 μM concentration of cytochalasin B shortens actin filaments by preventing addition of monomers to the growing end of the filament (Theodoropoulos et al., 1994). PLGA nanoparticles have been reported to be internalized into the cell by the endosomes. Following internalization, nanoparticles undergo a selective reversal of surface charge, interact with the endosomal membrane, and escape into the cytosol, resulting in release of entrapped drug inside the cell (Panyam et al., 2002). However, a high concentration of paclitaxel at the cell surface would also provide greater gradient to for passive diffusion of the drug across the cell membrane. In addition, a higher concentration of paclitaxel may saturate the efflux pumps resulting in greater intracellular accumulation of the drug. These observations are also supported by recent literature in which it has been reported that PLGA nanoparticles are not readily taken up by the cells, but rather deliver the drug locally (Xu et al., 2009). Therefore, it is hypothesized that, after intratumoral administration, nanoparticles may be internalized by the tumor cells whereas microparticles will release the encapsulated drug locally at the cell surface, which is taken up by the tumor cells by various mechanisms of transport.

5. Conclusion

Microparticles sustained the delivery of paclitaxel for 14 days, while nanoparticles rapidly released the drug completely in 24 h. Significantly higher cellular association of paclitaxel when delivered in microparticles was observed compared to nanoparticles or the solution. Consistent with these *in vitro* results, the 1 μm and 10 μm microparticles containing paclitaxel produced significantly higher anti-tumor activity *in vivo* compared to the nanoparticles or the commercial product, suggesting that a microparticle formulation of paclitaxel was significantly more effective delivery system than nanoparticles or commercial Paclitaxel Injection® given intravenously or intratumorally. The size-dependent cellular association of paclitaxel from PLGA nanoparticles and microparticles was sustained even as the incubation time of the particles with 4T1 cells was varied from 1 min to 5 h. At an incubation time of <1 min, microparticles produced significantly greater cellular association of paclitaxel compared to nanoparticles which strongly supports the hypothesis that the microparticles adhere to, and are retained in the mucus on the cell surface. Pre-treatment of nanoparticles and microparticles with porcine mucin significantly increased the cellular association of paclitaxel. Studies to investigate the mechanism of cellular association of particles confirmed that the particles are not internalized into the cells either by clathrin-mediated endocytosis or by macropinocytosis. This suggested that the only mechanism by which the cellular association of paclitaxel delivered in larger particles is enhanced is by non-specific adhesion and retention on the cell surface.

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