Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 65 (2007) 300–308
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

In vivo evaluation of a new polymer-lipid hybrid nanoparticle (PLN) formulation of doxorubicin in a murine solid tumor model

Ho Lun Wong [a], Andrew Mike Rauth [b], Reina Bendayan [a], Xiao Yu Wu [a,*]

[a] *Graduate Department of Pharmaceutical Sciences, University of Toronto, Toronto, Ont., Canada*
[b] *Experimental Therapeutics, Ontario Cancer Institute, Toronto, Ont., Canada*

Received 18 August 2006; accepted in revised form 24 October 2006
Available online 7 November 2006

Abstract

The purpose of this study is to evaluate the *in vivo* efficacy, unwanted toxicity and loco-regional distribution of a doxorubicin-loaded polymer-lipid hybrid nanoparticle (Dox-PLN) formulation in a murine solid tumor model after intratumoral injection. Dox-PLN were prepared by dispersing Dox in stearic acid and tristearin, with subsequent addition of a novel anionic polymer HPESO (hydrolyzed polymer of epoxidized soybean oil) to enhance the drug incorporation in the lipids. Solid tumors were obtained by injecting EMT6 mouse mammary cancer cells intramuscularly into the hind legs of BALB/c mice. Dox-PLN, blank PLN or surfactant formulations were injected intratumorally (IT) when tumors reached approximately 0.3 g. *In vivo* efficacy of treatment was measured by tumor growth delay (TGD), defined as the delay in time for the tumor to grow to 1.13 g relative to the untreated control. Signs of unwanted drug toxicity, the histology and morphology of tumor and heart tissues, and the IT distribution of Dox-PLN after IT treatment were examined or monitored. IT-administered Dox-PLN resulted in 70% and 100% TGD ($p < 0.01$) for Dox doses of 0.1 and 0.2 mg, respectively. Dox-PLN treated tumors developed substantially larger central necrotic regions than the untreated tumors, with Dox-PLN residues extensively distributed among the dead cell debris, suggesting that the anticancer effect of Dox-PLN was mainly a combined result of IT nanoparticle distribution and short-ranged, sustained drug release. Except for two of fifteen mice receiving the higher 0.2 mg Dox dose showing transient fur-roughing, all Dox-PLN treated mice showed no signs of toxicity. The present study demonstrates that Dox-PLN possess significant *in vivo* cytotoxic activity against solid tumors with minimal systemic toxicity. IT administered Dox-PLN have the potential to improve the therapeutic index of loco-regional solid tumor chemotherapy.

Keywords: Polymer-lipid hybrid nanoparticles; Doxorubicin; Cancer chemotherapy; *In vivo* efficacy

1. Introduction

The clinical success rate of chemotherapy is particularly low in the treatment of solid tumors [1]. The unique environment inside a solid tumor leads to a number of non-cellular drug resistance mechanisms. Irregularly developed intratumoral vasculature and the extracellular matrix of tumor cells both prevent efficient drug penetration into the tumor [2,3]. High interstitial pressures that can build up in solid tumors further exacerbate this problem [4,5]. In addition, the high acidity and low oxygen tension that are usually observed in a solid tumor may diminish the effectiveness of drugs that are basic and/or utilize oxygen free radicals for their anticancer action, e.g. doxorubicin (Dox) [6,7]. A substantial fraction of tumor cells in a solid tumor are in quiescent state [8]. This further compromises the therapeutic effectiveness of cancer chemotherapy for solid tumors, as many chemotherapeutic agents are more effective against rapidly dividing cells [9]. Because of these

Abbreviations: Dox, doxorubicin; IT, intratumoral; MMC, mitomycin C; PLN, polymer-lipid hybrid nanoparticles; SLN, solid lipid nanoparticles; TGD, tumor growth delay; TPL, tumor plus leg.

* Corresponding author. Graduate Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, 19 Russell Street, University of Toronto, Ont., Canada M5S 2S2. Tel.: +1 416 9785272; fax: +1 416 978 8511.
E-mail address: xywu@phm.utoronto.ca (X.Y. Wu).

0939-6411/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2006.10.022

in vivo non-cellular drug resistance mechanisms, drugs that are effective in cell lines *in vitro* do not necessarily work equally well against these cells when grown as solid tumors *in vivo*.

Local drug delivery systems offer promise for overcoming some of these obstacles to chemotherapy. In our previous study cross-linked sulphonylated microspheres containing mitomycin C (MMC) or Dox were directly delivered to the tumor by IT injection [10], an average of 79% and 185% increase in tumor growth delay compared to the control group were observed using MMC-loaded microspheres alone or combined with Dox-loaded microspheres, respectively. The normal tissue toxicity of drugs delivered *via* IT injected microsphere formulations was also significantly lower than that of the same amount of drug administered systematically [10]. Thus, the therapeutic index of MMC or MMC plus Dox chemotherapy is increased by local drug delivery *via* microsphere formulations.

The large size of microspheres, however, limits their ability to diffuse far away from the IT injection site. This may potentially reduce their effectiveness for loco-regional cancer therapy as many solid malignancies, such as breast cancer, tend to invade the surrounding tissues or metastasize to the loco-regional lymphatics. Compared to microspheres, submicron size particulate drug carrier may provide the necessary mobility to treat these types of loco-regional cancer involvement, as has been shown using various particulate carrier systems of submicron size [11–13].

It has been demonstrated that solid lipid nanoparticles (SLN), a submicron lipid-based formulation that can be used for delivery of several anticancer agents, can move far from the site of administration after they are administered intravenously [14,15], duodenally [16] or subcutaneously [17]. Significant drainage of inhaled SLN from the respiratory passage into the loco-regional lymph nodes has also been observed [18]. Compared to systemic drug administration, reports on the efficacy and side effects of SLN formulations of anticancer compounds, particularly those containing *in vivo* data, have so far been scarce. A study by Serpe et al. [19] showed that SLN carrying Dox, paclitaxel or cholesteryl butyrate were more effective than the conventional drug solutions in a human colorectal cancer cell line growing *in vivo*. In the limited *in vivo* efficacy data available [20], xenografted colorectal tumors were found to grow more slowly when treated with a SLN formulation loading with an analog of topoisomerase inhibitor. In brief, the findings reported to this point have supported the use of SLN for anticancer drug delivery, but it is also clear that more studies in this area are needed to further substantiate this claim.

Polymer-lipid hybrid nanoparticles (PLN) were devised on the basis of SLN. In comparison to other lipid-based formulations, incorporation of ionic polymers allows PLN to more efficiently encapsulate water-soluble, ionic compounds such as Dox hydrochloride. The loaded drugs were also shown to be more completely released. A typical PLN system can release 60–70% of its loaded drug instead of only 20–30% [21]. Co-encapsulation and simultaneous delivery of multiple drugs is also achievable, which adds further potential applications to this delivery system [21]. In addition, our previous studies showed that Dox-loaded PLN (Dox-PLN) were approximately 8 times more effective than free Dox solution in suppressing the growth of drug-resistant cancer cells *in vitro*, including both the murine EMT6 and human MDA435/LCC6 breast cancer cell lines [22,23]. Higher drug uptake and retention by those cell lines were also demonstrated [23]. It appeared that the small size of PLN made it possible for PLN to be internalized into the cancer cells, likely contributing to their superior *in vitro* efficacy compared to the free drug solution.

Based on these previous positive results, the present study was designed to evaluate whether the observed potent *in vitro* cytotoxicity of Dox-PLN is translatable into *in vivo* therapeutic effectiveness in the EMT6 solid tumor model. Tumor growth and general toxicity were monitored following IT injection of Dox-PLN. In addition, the tumoral distribution of PLN was examined. The present study provides evidence in support of the use of SLN-type systems for delivery of anticancer compounds to achieve better therapeutic outcomes.

2. Materials and methods

2.1. Materials

Dox (as a hydrochloride salt), stearic acid, tristearin and all other chemicals used, unless otherwise specified, were purchased from Sigma–Aldrich (Mississauga, ON, Canada). Stearic acid was purified by recrystallization from 95% ethanol. HPESO (hydrolyzed polymer of epoxidized soybean oil) anionic polymer was supplied by Drs. Z. Liu and S. Erhan (Food and Drug Administration, Washington, DC, USA) and Pluronic F68 (non-ionic block copolymer) was a kind gift from BASF Corp. (Florham Park, NJ, USA). The number and weight average molecular weights of HPESO used were determined by gel permeation chromatography and found to be $M_n = 3160$ and $M_w = 4866$. For details of polymer synthesis and characterization, please refer to Wong et al. [22].

2.2. Preparation of PLN containing Dox

To prepare Dox-PLN, a mixture of 70 mg of stearic acid, 30 mg of tristearin and 0.9 ml of an aqueous solution containing 7.5 mg of Dox and Pluronic-F68 (2.5% w/v) was warmed to 72–75 °C. Following the addition of 3.75 mg of HPESO polymer, the mixture was stirred for 10 min and subjected to ultrasonication for 3 min to form a lipid emulsion. The emulsion was dispersed in a 5% w/v dextrose + 2% w/v Pluronic F68 solution at 4 °C (1 part of emulsion to 4 parts of diluent) to form PLN. Free,

unloaded Dox was removed by gently stirring the Dox-PLN suspension with an excess amount of Sephadex C-25, a cationic ion-exchanger that effectively binds Dox [24]. Samples of Dox-PLN were drawn for Dox loading determination by UV–vis spectrophotometry and particle size measurement by photon correlation spectroscopy as previously described [21–23]. The Dox loading was approximately 5% and particle size averaged 290 nm in a typical Dox-PLN sample. Blank PLN were similarly prepared except that Dox was omitted.

2.3. Tissue cell culture

The murine breast carcinoma cell line EMT6/WT was kindly provided by Dr. Ian Tannock (Ontario Cancer Institute, Toronto, ON, Canada). Monolayers of cells (passages 5–30 in our hands) were cultured on 75-cm^2 polystyrene tissue culture flasks at 37 °C in 5% CO_2/95% air humidified incubator. Cancer cells were maintained in a pH 7.2 α-minimal essential medium (Ontario Cancer Institute Media Laboratory, Toronto, ON, Canada), supplemented with 10% fetal bovine serum (Cansera Inc., Etobicoke, ON, Canada). Cells grown to confluence (3–5 d after seeding, approximately 0.1 million of cells per dish) were trypsinized with 0.05% trypsin–EDTA (Invitrogen Inc., Burlington, ON, Canada), diluted (1/10) in a fresh growth medium and reseeded.

2.4. Evaluation of in vivo effectiveness and drug toxicity

All experiments and procedures used in the animal studies were approved by the Animal Use and Ethics Committee of the Ontario Cancer Institute. 8- to 10-week-old female BALB/c mice (BanHarbor, MA, USA) were inoculated intramuscularly in the left hind leg with 5×10^5 EMT6/WT cells in 50 μl of growth medium, one week after they were received. The tumor-plus-leg (TPL) diameter was monitored on a daily basis, after reaching 7.5 mm, with a plastic plate bearing a series of calibrated holes with diameters increasing in 0.5 mm increments. When the TPL diameters reached 8–9 mm (calibrated to be approximately 0.3 g tumor weight), typically 4–6 days after tumor cell inoculation, treatments were initiated.

Animals were randomized into groups containing 3–5 animals each. Each treatment group received 0.1 mg Dox in Dox-PLN, 0.2 mg Dox in Dox-PLN, or blank PLN, respectively. The control groups received a solution containing 5% w/v dextrose and 2% w/v Pluronic F68, or no treatment. Nanoparticle samples were diluted to the desired concentrations with the solution containing 5% w/v dextrose and 2% w/v Pluronic F68 that was pre-sterilized by filtration through a 0.22 μm filter. Overall, after taking the amount of Pluronic F68 initially present in the PLN formulations into consideration (2.5% Pluronic before dilution), treatments were found to contain 2–2.1% w/v Pluronic F68 after the dilution (control: 2%; 0.1 mg Dox in PLN group: 2.05%; Blank PLN and 0.2 mg Dox in PLN group: 2.1%). Each animal in the treatment groups was IT injected with 200 μl of the suspensions or the solution with a 26-gauge needle. TPL diameters were monitored daily. Mice were sacrificed when TPL diameters reached 13 mm, which was previously calibrated as 1.13 g tumor weight, or if the tumor never reached 13 mm in diameter, 20 days after treatment for humane purposes. At time of sacrifice tumors were excised and stored in 10% buffered formalin for gross morphological examination and histological study.

The efficacy of the treatments was reported in terms of normalized tumor growth delay (TGD) [10], according to the equation:

$$\mathrm{TGD} = (T_{\mathrm{treat}} - T_{\mathrm{control}})/T_{\mathrm{control}} \times 100\%$$

T_{treat} and T_{control} are referred as the mean number of days for the calibrated tumor weight to reach 1.13 g, for the treatment group and control group, respectively, where the day when the treatment started was assigned as Day = 0. Because in the initial trials, tumors treated with drug-free Pluronic/sucrose solution grew at the same rate (6.7 ± 0.6 days) as the untreated tumors (7.2 ± 3.2 days), untreated mice were directly used as the controls.

The toxicity of the treatment was evaluated in a semi-quantitative manner. Body weights of mice were measured daily for evaluation of the systemic toxicity of the treatments. A general toxicity score was assigned to each mouse as previously described [10] with some modifications (0 = no weight loss; 1 = 1–2 g weight loss; 2 = 2 g or more weight loss). Only the lowest body weight loss (compared to the body weight on Day 0) within 5 days after the treatment was considered because some mice actually lost weight as their tumors grew in size. Body weight loss greater than 20% was regarded as a sign of significant systemic toxicity and the mice were sacrificed for humane purposes. Other signs of unwanted toxicity monitored included fur-roughing and shedding, local trauma at site of injection and decreases in general animal activity. The hearts of selected mice were preserved after they were sacrificed to allow histological evaluation of possible cardiotoxicity of Dox-PLN.

2.5. Morphological and histological examination

Each excised tumor was dissected into halves along the longitudinal mid-line (i.e. along the longest dimension of the tumor). The interior sections of tumor were digitally photographed under an optical microscope. Selected tumor tissue samples were also sent to the University Health Network Clinical Research Program (Toronto General Hospital, ON, Canada) for hematoxylin and eosin (H&E) staining and slide preparation. The stained slides were digitally scanned and the images were enhanced using a computer software system (ImageScope Virtual Slide, version 6.25, Aperio Technol., Vista, CA, USA) for histological examination.

2.6. Data analysis

Results are presented as means ± SD from a minimum of three separate experiments unless otherwise specified. Log–Rank tests were conducted to compare the effects of different treatments to the controls. In all analyses, a value of $p < 0.05$ was considered significant.

3. Results

3.1. Efficacy of intratumorally (IT) administered Dox-PLN

The effect of Dox-PLN treatment on *in vivo* tumor growth is presented in Fig. 1. In Fig. 1A, the results pooled from all runs are expressed in terms of fractions of mice that survived (mice with tumors remaining smaller than the cutoff size (TPL diameter <13 mm)) as a function of time after treatment. In Fig. 1B and C, the changes in tumor weight from selected individual mice (blank PLN and Dox-PLN) are shown as a function of time before and after receiving treatment (treatment delivered on day 0) are plotted. Data for only one representative group (total of five mice) of each treatment are presented in the profiles for clarity. Statistical data for all animals are summarized in Table 1. In brief, mice receiving 0.1 or 0.2 mg dose of Dox in the form of Dox-PLN demonstrated significant delay in tumor growth ($p < 0.005$) when compared to the mice receiving Pluronic/dextrose or blank PLN. The mean times for the tumor to reach the cutoff (13 mm TPL diameter or 1.13 g tumor weight) were significantly prolonged by 5–7 days, which translates into TGD values of 71% and 100% in mice treated with 0.1 and 0.2 mg Dox as Dox-PLN, respectively. This finding is likely not caused by the skewing effect of a few extreme values as the mean and median values are very close (Table 1). It should also not be caused by Pluronic F68 itself as tumor growth was not suppressed by blank PLN). The higher dose of 0.2 mg Dox per mouse appears to be more effective than 0.1 mg Dox per mouse. It took approximately 2 more days to reach the cutoff point. However, the difference between these two doses was not statistically significant. Some tumors treated with Dox-PLN actually regressed for a few days before they grew again (Fig. 1C).

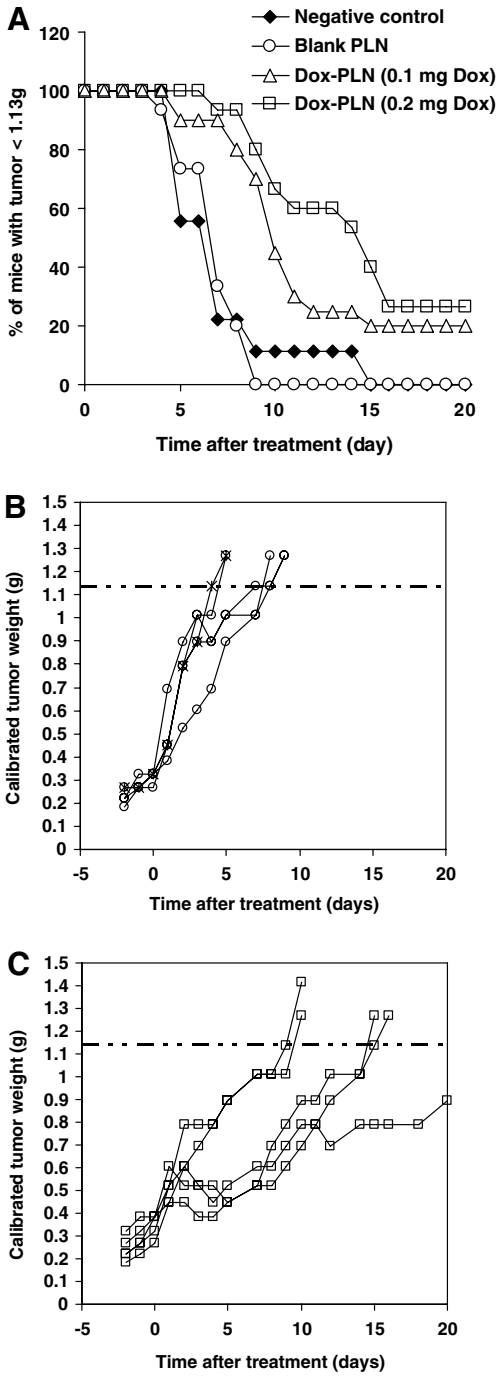

Fig. 1. Effect of Dox-PLN treatment on EMT6/WT tumor growth in BALB/c mice. (A) presents the fractions of surviving mice (mice with tumor below the cutoff weight (1.13 g)) after treatment; (B) and (C) show typical time profiles of calibrated tumor weight before and after treatment in individual mice sampled from (B) blank PLN group and (C) Dox-PLN (0.2 mg Dox) group. Mice were inoculated with EMT6/WT cells intramuscularly in the hind leg and treatments were injected into the tumor when it reached approximately 0.3 g. Treatments tested included 5% dextrose + 2% Pluronic F68 (negative control), blank PLN, 0.1 mg of Dox as Dox-PLN, 0.2 mg of Dox as Dox-PLN. In all figures, Day 0 is referred to the day when the mice received treatments. Mice were sacrificed when their tumors reached 1.13 g in weight or 20 days after treatment.

3.2. General toxicity of it administered Dox-PLN treatment

Fig. 2A–D present representative time profiles of the body weight of the mice before and after receiving treatment. None of the mice died or lost 10% body weight or more before the tumors reached the cutoff size. In general, treatment with Dox-PLN or blank PLN resulted in over 1 g body weight loss only in a few mice between day 0 and day 5, and these losses all recovered as the study progressed. The toxicity scores were 0.33 ± 0.50, 0.20 ± 0.41, 0.15 ± 0.37, 0.40 ± 0.63 (mean ± SD) in untreated mice, blank PLN group, Dox-PLN (0.1 mg Dox) group and Dox-PLN (0.2 mg Dox) group, respectively. No significant differences in the scores among different groups were detected. The finding indicates a lack of general toxicity caused by the blank PLN and the Dox-PLN formulations.

Table 1
Summary of the effect of Dox-PLN against tumor growth in BALB/c mice

Treatment	Number of experiments × number of subjects	Mean TGD (SD), days	Median TGD, days	Normalized TGD	*p* value (vs. no treatment)	*p* value (vs. blank PLN)
No treatment	3 × 3	7.2 (3.2)	7	N/A	N/A	0.95
Blank PLN	3 × 5	6.9 (1.6)	7	N/A	0.95	N/A
0.1 mg Dox as Dox-PLN	4 × 5	12.3 (4.6)	11	70.8%	0.0033*	0.0006*
0.2 mg Dox as Dox-PLN	3 × 5	14.4 (4.5)	15	100%	0.0003*	0.0001*

Results were expressed in terms of number of days required for the tumor weight to reach 1.13 g. Tumors that never reached the cutoff weight of 1.13 g within 20 days after treatment, for statistical analysis they were arbitrarily assigned 20 days duration. *p* values were calculated based on the χ^2 values obtained from Log to Rank tests comparing the data of the various treatments presented in Fig. 1A. The *p* value comparing the 0.1 mg Dox group to 0.2 mg Dox group is 0.294 (not significant). N/A, not applicable; TGD, tumor growth delay.
* Statistically significant (*p* value < 0.05 relative to control).

Except for two of the mice treated with 0.2 mg Dox showing moderate fur-roughing, none of the tested mice manifested signs of other adverse effects (inactivity, tissue damage at injection site) as specified in the method section.

3.3. Gross and histological examination of tumor tissues

Both gross and histological examinations were performed to investigate the internal morphology of tumor tissues. Microscopic photographs of representative samples of tumors excised from mice and sectioned through the middle from each treatment group is shown in Fig. 3A–D. The tumor treated with blank PLN (Fig. 3A) was solid throughout the tumor except for a small necrotic region at the center. Tumor tissues treated with different doses of Dox-PLN (Fig. 3B–D) appear looser and irregular towards the center of the tumor. The tumor treated with a higher dose (0.2 mg Dox) of Dox-PLN (Fig. 3C) seems to have more necrosis than that with the lower Dox dose (0.1 mg Dox) (Fig. 3B). However, Fig. 3D shows the same treatment dose, i.e. 0.2 mg Dox, could result in less necrosis when the Dox-PLN were injected in the tumor capsule and stayed there (Fig. 3D).

To examine the histology of the treated tumors, and the interactions between the cancer cells and Dox-PLN *in vivo*, selected tumor samples were H&E stained and examined.

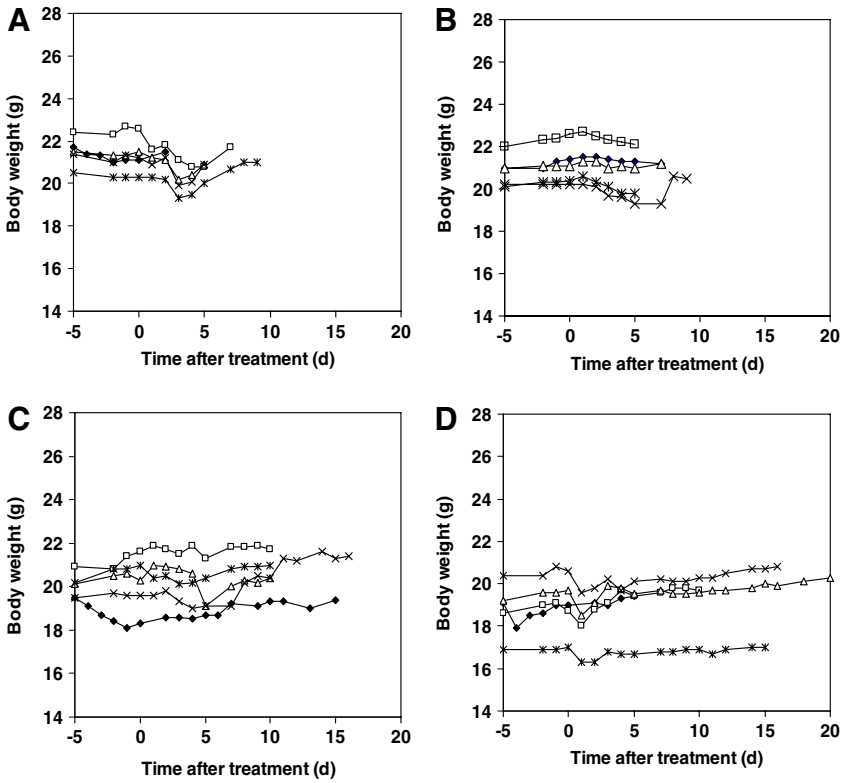

Fig. 2. Time profiles of body weight of tumor-bearing mice treated with (A) negative control (dextrose 5% + 2% Pluronic F68); (B) blank PLN; (C) 0.1 mg Dox in form of Dox-PLN; (D) 0.2 mg Dox in form of Dox-PLN. BALB/c mice were inoculated with EMT6/WT tumor in the hind leg and received treatment 5 days later. Day 0 in the graphs represents the day of treatment and each curve represents the body weight time profile of a single mouse.

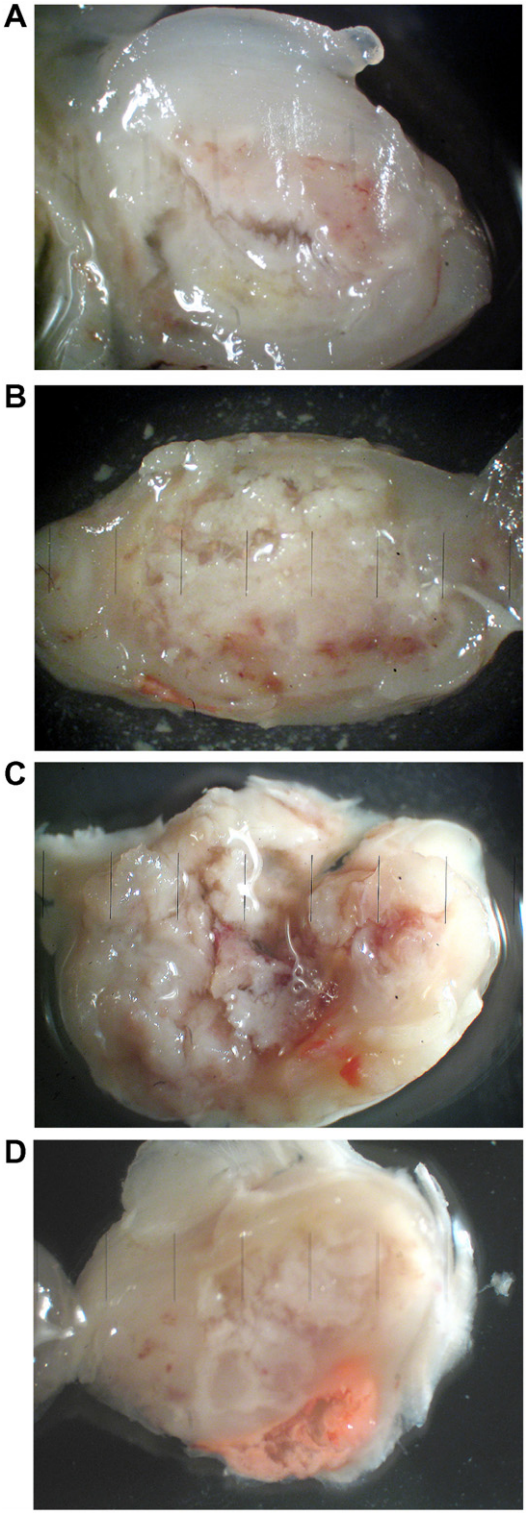

Fig. 3. Effect of Dox-PLN on the morphology of EMT6 tumors grown in BALB/c mice. Treatments included (A) blank PLN; (B) 0.1 mg Dox as Dox-PLN; (C) and (D) 0.2 mg Dox as Dox-PLN were administered intratumorally to the EMT6 tumors grown in the hind legs of BALB/c mice. Tumors that grew to 13 mm in diameter or larger were excised and preserved in 10% buffered formalin. Cross-sectioning along the longitudinal midline of each tumor was performed to expose their internal structure. Each interval between the graduated marks in the pictures represents 2 mm.

Fig. 4 shows a tumor sample treated with Dox-PLN containing 0.2 mg Dox (the same tumor as shown in Fig. 3C was chosen for comparison). The paler, looser regions primarily consist of cancer cells that are in the process of necrosis. The globular, pink tissues at the perimeter are the leg muscles. When compared to Fig. 3C, it is evident that the large regions of rough, loose tissues observed in gross examination are in fact necrotic cancer cells.

Fig. 4B shows the magnified view of a part of the peripheral tumor region (Black rectangle in Fig. 4A). There is extensive infiltration of EMT6 tumor cells into the muscular tissues, which confirms the aggressiveness of the EMT6 tumor cells shown previously [10]. Dox-PLN and their aggregates were observed near the center of the picture (pink particulate matter identified by arrows). There are two notable findings. Dox-PLN do not just stay on the surface layers of cancer cells, but also distribute to cells that are several layers deep. In addition, the cancer cells that are located near the Dox-PLN are generally looser and disintegrated, whereas cancer cells relatively far from Dox-PLN (to the left and the right in Fig. 4B) appear more intact and healthier. Similar findings are demonstrated in Fig. 4C, which presents a part of the central, necrotic region of the tumor (region in dotted rectangle of Fig. 4A). The residual Dox-PLN are dispersed within several layers of loose, necrotic cancer cells (on the right side of the image indicated by arrows). These images suggest that tumor necrosis may be caused or accelerated by Dox-PLN. Extensive tumor necrosis may be the result of movement of Dox-PLN within the tumor structure.

4. Discussion

Cytotoxic anticancer drugs that are effective *in vitro* are not necessarily effective *in vivo*. For example, wild-type EMT6 cells have been shown to be relatively sensitive to Dox when cultured as monolayers in a number of previous studies from our laboratory (e.g. [24,25]). The D_{10} value (drug concentration that kills 90% cells) of Dox in EMT6/WT cell line can be as low as 1 μg/mL [25]. However, this is not translated well in *in vivo* studies. Our group previously attempted a test of the efficacy of free Dox using the same animal tumor model. Because of severe tissue necrosis at the injection sites and high toxicity, we dropped this arm of the study. When Dox microsphere formulation was administered IT, it was only marginally effective with up to 32% TGD resulted [26]. This actually prompted Cheung et al. [10] to explore the use of microspheres for combinational therapy (MMC plus Dox) to enhance *in vivo* cytotoxicity. It is therefore exciting to find that Dox-PLN result in significant growth delay in a tumor model that is supposedly refractory to Dox treatment even with IT injection of Dox-loaded microspheres.

It should be noted that the TGD method used in this study may not necessarily fully demonstrate the anticancer

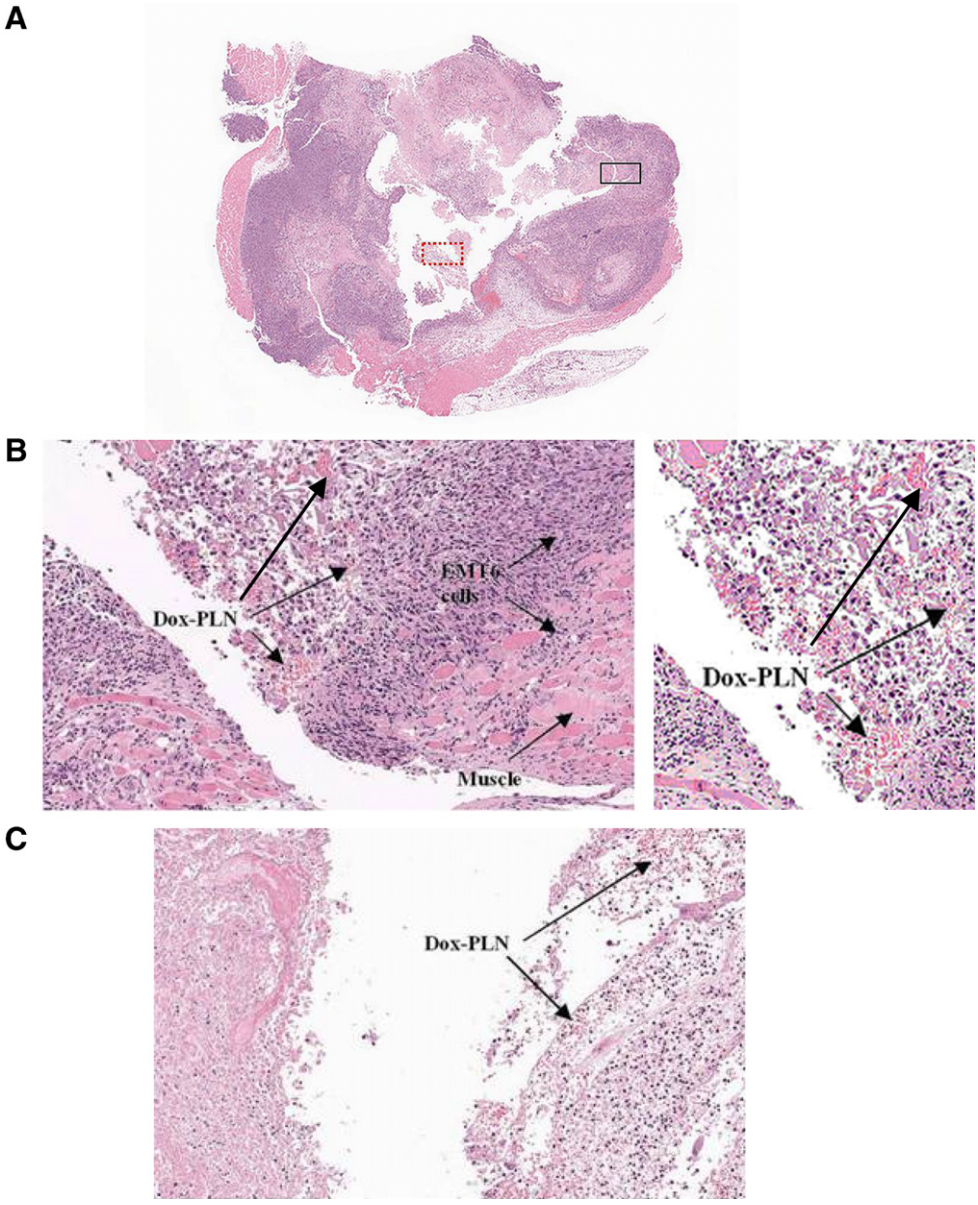

Fig. 4. Histology of a representative sample of tumor treated with Dox-PLN. A sample of tumor treated with Dox-PLN containing 0.2 mg Dox was excised and H&E stained. Portions of (A) were digitally enhanced 10× using a computer imaging software package (Scanscope Virtual Slide) and presented in (B) (peripheral portion, black rectangle in (A)) and (C) (central portion, red rectangle in A). In (B) and (C), arrows point to Dox-PLN and their aggregates remaining in the tumor.

activity of the tested agents. TGD are calculated based on the length of time for the tumor to reach a predetermined cutoff TPL diameter, which is correlated to the tumor size. However, as shown in Fig. 3B and C, enhanced necrosis, induced by IT injected Dox-PLN, occurred mainly at the centers of the tumors, whereas the tumor capsules are left essentially intact and are likely actively growing. Growth delay due to a moderate tumor cell killing effect of treatment, even it is significant, may not be indicative of the detailed effects of treatment. It is therefore useful to complement this method with the studies of the gross morphology of the treated tumors. In the present studies, both approaches indicate that Dox-PLN are effective against breast cancer cells *in vivo*.

The studies of tumor morphology and histology provide a hint on the mechanisms of the tumor growth suppression mediated by Dox-PLN. First of all, TGD was not caused by either the nanoparticle lipids or the surfactants used in the PLN, as the activity of blank PLN was similar to the no treatment control, and little cell damage was also seen in the gross examination of tumor treated with blank PLN (Fig. 3A). In contrast, as shown in Fig. 4B and C, necrosis regions are typically found proximal to the residual Dox-PLN distributed within the tumor samples viewed 20 days post-injection. This shows that the anticancer effect of Dox-PLN is relatively short-ranged, and their direct physical contact to cancer cells likely results in the strongest anticancer effect. It is there-

fore critical to achieve reasonable IT dispersion of the drug carriers. When injected via the IT route, Dox-PLN were seen to be distributed fairly extensively within the tumor (e.g. Fig. 4). This can substantially reduce the distances between the tumor cells and Dox-PLN, and allow more extensive tumor cell kill (Fig. 3B) to occur. This is evident when compared to specific tumor samples in which Dox-PLN were injected to the highly dense capsule regions of the tumor and were subsequently trapped and did not spread out (e.g. Fig. 3D). In those samples only moderate tumor necrosis was observed. The relatively good IT distribution may be one of the reasons that contribute to the superior *in vivo* efficacy of Dox-PLN over Dox-loaded microsphere (Dox-MS) systems, which we did not observe substantial particle distribution away from the site of injection. In addition, the enhanced cellular uptake and retention of Dox by PLN may also play a role in the higher efficacy of Dox-PLN than Dox-MS.

It must be noted that some extent of tumor necrosis nonetheless occurred in cells distant to the particles (e.g. Fig. 3D), so a part of cell killing could still be caused by the free drug molecules released and diffused from Dox-PLN. This relatively long-ranged anticancer activity is needed because it obviates the need for 100% particle delivery to the cancer cells for complete cell kill. However, the diffusion of Dox molecules through multicellular layers is reportedly very slow [27]. The inefficiency of this process is likely compensated by the use of IT-administered Dox-PLN for Dox delivery as this approach may provide extended time for the Dox molecules to diffuse intratumorally. In summary, the present findings are in general agreement with those obtained in our previous *in vitro* studies of Dox-PLN [22,23], which revealed a mixed mechanism that combines endocytosis of Dox-PLN by cancer cells and cytotoxicity due to the free, released drug molecules.

Overall, the normal tissue toxicity of Dox-PLN was quite minimal. In fact, the general toxicity scores of Dox-PLN treated mice were similar to the control group. It has been shown in BALB/c mice the LD_{50} (the drug dose that results in death of one-half of the tested animals) value for Dox administered by intraperitoneal injection was 20.6 mg Dox/kg body weight [28]. In the present study, all of the mice treated with 0.2 mg of Dox (equivalent to 10 mg/kg dose for a 20 g mouse) survived until sacrificed when the tumors reached the growth cutoff. In addition, no unusual morphology was detected in the heart tissue samples of mice 5–7 days after Dox-PLN treatment as determined by the pathologists at the Ontario Cancer Institute (data not shown). Treatment cardiotoxicity can be more accurately assessed with larger number of heart samples and a more specialized method (e.g. examination of the left ventricular function). Higher doses and multiple doses of Dox-PLN should be tested in the future. The present results nonetheless indicate that single-dose IT treatment with Dox-PLN may induce less unwanted *in vivo* side effects than free Dox solution.

Finally, during the study, we also noticed that IT injected Dox-PLN could be observed in the proximal inguinal lymph node (data not shown). This finding is consistent with the basic principle of sentinel lymph node mapping, which assumes that submicron particulate matter will naturally drain to the loco-regional lymphatic system [29]. It also indicates that even though tumor capsules are fairly intact, there may be some anatomical defects that allow nanoparticles to "escape" from the center of the tumor. Currently, more in-depth studies focusing on lymphatic drainage of PLN are being conducted by our group. These studies may help to further understand the therapeutic value of loco-regional cancer chemotherapy using lipid nanoparticles.

5. Conclusion

The present study demonstrates that the new PLN delivery system loaded with Dox is effective for tumor treatment in a well-established animal model. Significant tumor growth delay and tumor necrosis were observed in tumors treated with IT injected Dox-PLN, probably caused by a mixed mechanism that involves direct cell-particle contact effects and intratumoral diffusion of free drug. The unwanted normal tissue toxicity of this form of localized treatment is very low. Initial studies also indicate that the nanoparticles may spread to the regional lymph nodes when administered intratumorally. The findings suggest that PLN carrying anticancer agents may be useful for loco-regional treatment of breast cancer with an improved therapeutic index.

Acknowledgments

The authors thank the Canadian Institutes of Health Research for supporting this project, Dr. I. Tannock for providing the EMT6 cancer cell line, Drs. Z. Liu and S. Erhan for the HPESO polymer. We also want to gratefully acknowledge Mr. R. Kuba from Ontario Cancer Institute for his technical assistance in animal handling, and the Canadian Graduate Scholarship from Natural Sciences and Engineering Research Council of Canada and Ben Cohen Fund to H.L. Wong.

References

[1] I.F. Tannock, C.M. Lee, J.K. Tunggal, D.S. Cowan, M.J. Egorin, Limited penetration of anticancer drugs through tumor tissue: a potential cause of resistance of solid tumors to chemotherapy, Clin. Cancer Res. 8 (2002) 878–884.

[2] A. Bhattacharya, K. Toth, R. Mazurchuk, J.A. Spernyak, H.K. Slocum, L. Pendyala, R. Azrak, S. Cao, F.A. Durrani, Y.M. Rustum, Lack of microvessels in well-differentiated regions of human head and neck squamous cell carcinoma a253 associated with functional magnetic resonance imaging detectable hypoxia, limited drug delivery, and resistance to irinotecan therapy, Clin. Cancer Res. 10 (2004) 8005–8017.

[3] B. St. Croix, R.S. Kerbel, Cell adhesion and drug resistance in cancer, Curr. Opin. Oncol. 9 (1997) 549–556.
[4] R.K. Jain, Transport of molecules in the tumor interstitium: a review, Cancer Res. 47 (1987) 3039–3051.
[5] M.J. Boyer, I.F. Tannock, Cellular and molecular basis of chemotherapy, in: I.F. Tannock, R.P. Hill (Eds.), The Basic Science of Oncology, McGraw-Hill, Toronto, 1998, pp. 350–369.
[6] J.A. Kellen, Multidrug resistance in human malignancies, in: Reversal of Multidrug Resistance in Cancer, CRC Press, Ann. Arbor, 1993, pp. 69–91.
[7] I.F. Tannock, D. Rotin, Acid pH in tumors and its potential for therapeutic exploitation, Cancer Res. 49 (1989) 4373–4384.
[8] S. Rockwell, C.S. Hughes, Effects of mitomycin C and porfiromycin on exponentially growing and plateau phase cultures, Cell Prolif. 27 (1994) 153–163.
[9] J.M. Slingerland, I.F. Tannock, Cell proliferation and cell death, in: I.F. Tannock, R.P. Hill (Eds.), The Basic Science of Oncology, McGraw-Hill, Toronto, 1998, pp. 134–165.
[10] R.Y. Cheung, A.M. Rauth, X.Y. Wu, In vivo efficacy and toxicity of intratumorally delivered mitomycin C and its combination with doxorubicin using microsphere formulations, Anticancer Drugs 16 (2005) 423–433.
[11] A. Hagiwara, C. Sakakura, M. Shirasu, T. Togawa, Y. Sonoyama, J. Fujiyama, Y. Ebihara, T. Itoh, H. Yamagishi, Intraperitoneal injection of dextran sulfate as an anti-adherent drug for the prevention of peritoneal metastasis of cancer shows low toxicity in animals, Anticancer Drugs 11 (2000) 393–399.
[12] S.M. Moghimi, R.R. Rajabi-Siahboomi, Advanced colloid-based systems for efficient delivery of drugs and diagnostic agents to the lymphatic tissues, Prog. Biophys. Mol. Biol. 65 (1996) 221–249.
[13] F. Ikomi, G.K. Hanna, G.W. Schmid-Schonbein, Size- and surface-dependent uptake of colloid particle into the lymphatic system, Lymphology 32 (1999) 90–102.
[14] G.P. Zara, R. Cavalli, A. Bargoni, A. Fundaro, D. Vighetto, M.R. Gasco, Intravenous administration to rabbits of non-stealth and stealth doxorubicin-loaded solid lipid nanoparticles at increasing concentrations of stealth agent: pharmacokinetics and distribution of doxorubicin in brain and other tissues, J. Drug Target. 10 (2002) 327–335.
[15] A. Fundaro, R. Cavalli, A. Bargoni, D. Vighetto, G.P. Zara, M.R. Gasco, Non-stealth and stealth solid lipid nanoparticles (SLN) carrying doxorubicin: pharmacokinetics and tissue distribution after IV administration to rats, Pharm. Res. 42 (2000) 337–343.
[16] G.P. Zara, A. Bargoni, R. Cavalli, A. Fundaro, D. Vighetto, M.R. Gasco, Pharmacokinetics and tissue distribution of idarubicin-loaded solid lipid nanoparticles after duodenal administration to rats, J. Pharm. Sci. 91 (2002) 1324–1333.
[17] L.H. Reddy, R.K. Sharma, K. Chuttani, A.K. Mishra, R.S.R. Murthy, Influence of administration route on tumor uptake and biodistribution of etoposide loaded solid nanoparticles in Dalton's lymphoma tumor bearing mice, J. Control. Release 105 (2005) 185–198.
[18] M.A. Videira, M.F. Botelho, C. Santos, L.F. Gouveia, J.J. Pedroso de Lima, A.J. Almeida, Lymphatic uptake of pulmonary delivered radiolabelled solid lipid nanoparticles, J. Drug Target. 10 (2002) 607–613.
[19] L. Serpe, M.G. Catalano, R. Cavalli, E. Ugazio, O. Bosco, R. Canaparo, E. Muntoni, R. Frairia, M.R. Gasco, M. Eandi, G.P. Zara, Cytotoxicity of anticancer drugs incorporated in solid lipid nanoparticles on HT-29 colorectal cancer cell line, Eur. J. Pharm. Biopharm. 58 (2004) 673–680.
[20] J. Williams, R. Lansdown, R. Sweitzer, M. Romanoswki, R. Labell, R. Ramaswami, E. Unger, Nanoparticle drug delivery system for intravenous delivery of topoisomerase inhibitors, J. Control. Release 91 (2003) 167–172.
[21] H.L. Wong, R. Bendayan, A.M. Rauth, X.Y. Wu, Development of solid lipid nanoparticles containing ionically complexed chemotherapeutic drugs and chemosensitizers, J. Pharm. Sci. 93 (2004) 1993–2004.
[22] H.L. Wong, A.M. Rauth, R. Bendayan, X.Y. Wu, A new polymer-lipid hybrid nanoparticle system increases cytotoxicity of doxorubicin against multidrug resistant breast cancer cells, Pharm. Res. 23 (2006) 1574–1585.
[23] H.L. Wong, R. Bendayan, A.M. Rauth, M. Ramaswamy, H.Y. Xue, X.Y. Wu, A mechanistic study of enhanced doxorubicin uptake and retention in multidrug resistant breast cancer cells using a polymer-lipid hybrid nanoparticle (PLN) system, J. Pharmcol. Exp. Ther. 317 (2006) 1372–1381.
[24] Z. Liu, R. Cheung, R. Bendayan, J. Ballinger, M. Rauth, X.Y. Wu, A study of doxorubicin loading onto and release from sulfopropyl dextran ion-exchange microspheres, J. Control. Release 77 (2001) 213–224.
[25] R.Y. Cheung, A Study of Alternative Delivery Strategies and Systems to Enhance the Therapeutic Effect and Cytotoxic Activity of Anticancer Agents (PhD Thesis). University of Toronto, Ontario, 2005, pp. 254–255.
[26] Z. Liu, J. Ballinger, M. Rauth, R. Bendayan, X.Y. Wu, Delivery of an anticancer drug and a chemosensitizer to murine breast sarcoma by intratumoral injection of sulfopropyl dextran microspheres, J. Pharm. Pharmacol. 55 (2003) 1063–1073.
[27] J.K. Tunggal, D.S.M. Cowan, H. Shaikh, I.F. Tannock, Penetration of anticancer drugs through solid tissue: a factor that limits the effectiveness of chemotherapy for solid tumors, Clin. Cancer Res. 5 (1999) 1583–1586.
[28] M. Yoshida, Y. Maehara, K. Sugimachi, MST-16, a novel bis-dioxopiperazine anticancer agent, ameliorates doxorubicin-induced acute toxicity while maintaining antitumor efficacy, Clin. Cancer Res. 5 (1999) 4295–4300.
[29] P.J. Tanis, O.E. Nieweg, R.A.V. Valdes Olmos, E.J. Th Rutgers, B.B.R. Kroon, History of sentinel node and validation of the technique, Breast Cancer Res. 3 (2001) 109–112.