Current Drug Delivery, 2018, *15*, 1261-1270

RESEARCH ARTICLE

Optimizing Antitumor Efficacy and Adverse Effects of Pegylated Liposomal Doxorubicin by Scheduled Plasmapheresis: Impact of Timing and Dosing

Romeo Ngoune[1], Christine Contini[1], Michael M. Hoffmann[1], Dominik von Elverfeldt[2], Karl Winkler[1] and Gerhard Pütz[1,*]

[1]*Medical Center - University of Freiburg, Faculty of Medicine, Institute for Clinical Chemistry and Laboratory Medicine, Freiburg, Germany;* [2]*Medical Center - University of Freiburg, Faculty of Medicine, Department of Diagnostic Radiology Medical Physics, Freiburg, Germany*

ARTICLE HISTORY

Received: December 01, 2017
Revised: April 11, 2018
Accepted: May 11, 2018

DOI:
10.2174/1567201815666180518125839

Abstract: ***Background*:** Nanoscale drug delivery systems accumulate in solid tumors preferentially by the enhanced permeation and retention effect (EPR-effect). Nevertheless, only a miniscule fraction of a given dosage reaches the tumor, while >90% of the given drug ends up in otherwise healthy tissues, leading to the severe toxic reactions observed during chemotherapy. Once accumulation in the tumor has reached its maximum, extracorporeal elimination of circulating nanoparticles by plasmapheresis can diminish toxicities.

***Objective*:** In this study, we investigated the effect of dosing and plasmapheresis timing on adverse events and antitumor efficacy in a syngeneic rat tumor model.

***Methods*:** MAT-B-III cells transfected with a luciferase reporter plasmid were inoculated into female Fisher rats, and pegylated liposomal doxorubicin (PLD) was used for treatment. Plasmapheresis was performed in a discontinuous manner *via* centrifugation and subsequent filtration of isolated plasma.

***Results*:** Bioluminescence measurements of tumor growth could not substitute caliper measurements of tumor size. In the control group, raising the dosage above 9 mg PLD/kg body weight did not increase therapeutic efficacy in our fully immunocompetent animal model. Plasmapheresis was best done 36 h after injecting PLD, leading to similar antitumor efficacy with significantly less toxicity. Plasmapheresis 24 h after injection interfered with therapeutic efficacy, while plasmapheresis after 48 h led to fewer side effects but also to increased weight loss.

***Conclusion*:** Long-circulating nanoparticles offer the unique possibility to eliminate the excess of circulating particles after successful accumulation in tumors by EPR, thereby reducing toxicities and likely toxicity-related therapeutic limitations.

Keywords: Cancer therapy, EPR-effect, liposomes, pegylated liposomal doxorubicin, toxicity, adverse events, plasmapheresis.

1. INTRODUCTION

Although anticancer therapy has been improved by several new therapeutic approaches in recent years, chemotherapy remains an important workhorse in the battle against cancer. To reduce the acute adverse effects of chemotherapeutic drugs, nanoscale particle-based drug delivery systems (DDS) are a very promising approach [1]. While liposomes were already used as a "DDS prototype" decades ago [2], several new materials ranging from simple polymeric micelles to macromolecular superstructures have proven successful for drug delivery [3, 4]. A new generation of "smart" DDS allowing controlled release at the target site has been developed to overcome the paradox of stable drug entrapment during circulation but efficient release once the target tissue is reached [5]. A vast variety of ligands have been used to specifically target cancer cells [1], but when treating solid cancers, almost all DDS accumulate *via* the enhanced permeation and retention (EPR) effect, with leaky endothelial structures the preferred entry gate [6, 7]. Most successful DDS-based nanomedicine is pegylated liposomal doxorubicin (PLD), used for many years now in clinical practice [8]. While chemotherapy with free doxorubicin is limited by its severe cardiotoxicity, PLD inflicts minimal cardiotoxicity. Unfortunately, new adverse effects like skin toxicity and mucositis have occurred, limiting the use of

*Address correspondence to this author at the Medical Center - University of Freiburg, Faculty of Medicine, Institute for Clinical Chemistry and Laboratory Medicine, Freiburg, Germany; Tel: ++49-761-270-32070; Fax: ++49-761-270-34440; E-mail: Gerhard.puetz@uniklinik-freiburg.de

1875-5704/18 $58.00+.00

PLD [9]. Long-circulating nanoparticles like PLD accumulate *via* the EPR effect [7]. Besides site-specific enhanced accumulation, this specific route of distribution offers the unique opportunity to diminish DDS toxicities *via* scheduled plasmapheresis [10]. We recently showed that liposome accumulation in tumor tissue is a route of no return, and a plasmapheresis-induced drop in liposomal plasma concentration does not affect the concentration of liposomes in the tumor [11]. In contrast to tumor tissue, scheduled plasmapheresis leads to a significant lower accumulation of liposomes in skin and animals' paws [11]. Plasmapheresis of natural nanoscale lipoprotein particles is a highly sophisticated technology applied in clinical practice for decades [12]. In clinical pilot studies, double filtration plasmapheresis was successfully used in combination with PLD, and respective chemotherapy already demonstrated very mild adverse events profiles [13, 14]. To enable the broader clinical use of this unique concept, we need evidence that scheduled plasmapheresis allows fewer side effects while preserving antitumor efficacy. Within this paper, we address the impact of dosing and plasmapheresis timing on side effects and antitumor efficacy in a small animal model. Bioluminescence imaging using firefly luciferase has been suggested to monitor the tumor response to chemotherapy noninvasively [15] and was evaluated as a monitoring tool for anticancer efficacy as well.

2. METHODS

2.1. Materials

PLD (DOXOVES®) was purchased from FormuMax Scientific Inc. (Sunnyvale, CA, USA). Luciferin was provided by Invitrogen (Karlsruhe, Germany).

2.2. Characterization of Liposomes

Liposomes were characterized by photon correlation spectroscopy (Nicomp Submicron Particle Analyser Model 380) to determine their mean size, size distribution, and polydispersion index (PDI). Hydrogenated soy phosphatidylcholine (HSPC) concentration was quantified according to Stewart [16].

2.3. Cell Culture

MAT-B-III Cells (ATCC® CRL-1666™, American Type Culture Collection, Manassas, USA) were cultured in Iscove basal medium (Biochrom, Berlin, Germany) supplemented with 10% Fetal Calf Serum (Biochrom) at 37°C in a humidified incubator with 5% CO_2 atmosphere. The medium was replaced every 6 days, and the cells seeded into a new culture dish when a confluence of 80% was attained.

2.4. Animal Study

Animal experiments were performed according to national and international guidelines (EU Directive 2010/63/EU) and approved by the local authority (reference G07/60 and G12/09). Female Fischer's rats F344/DuCrl (160 - 200 g) were purchased from Charles River (Sulzfeld, Germany). Rats were fed *ad libitum*. Temperature and relative humidity were kept constant at 20 – 21°C and 60%, respectively. For all animal studies, human care was given to the animals, and all animals were euthanized at the end of experiments. Syngeneic MAT-B-III rat mammary tumor cells were trypsinized and resuspended in PBS, and 2 x 10^5 cells in 100 µl PBS were inoculated subcutaneously in the flank of female Fischer rats to generate tumors.

2.5. Measurement of Plasma Elimination of PLD

Blood was withdrawn from the tail vein and 10 µl serum were diluted with 90 µl H_2O. By adding 10 µl of 3 M trichloroacetic acid, protein was precipitated and liposomal doxorubicin was liberated. After centrifugation (5 min at 18000g), the fluorescence intensity of 70 µl of the clear supernatant was measured in a precision cuvette at 470 nm/555 nm (exc./em.) on a luminescence spectrometer LS 50 (Perkin Elmer). A spiked pool of human plasma was used for calibration; a typical calibration curve is shown in supplemental Fig. (**S4**).

2.6. Luciferase Transfection

Transfection was performed using Nanofectin (PAA, Coelbe, Germany) according to the product manual. MAT-B-III cells were cultivated in a 25 cm^2 flask to 50% confluence and were transfected with pGL3 control vector for luciferase expression and pcDNA3-plasmid (Thermo Fisher) to mediate geneticin resistance. To 245 µl 150 mM NaCl 2.5 µl of each plasmid solution (1µg/µl) were given, and this solution was added to a mixture of 16 µl nanofectin and 234 µl 150 mM NaCl. After mixing for 30 min at RT, the mixture was added to the cells, growing in serum-free media. After 48 h, successfully transfected cells were selected by adding geneticin G-418 (0.5 mg/ml, Thermo Fisher) to the cell culture media.

Luciferase expression was confirmed prior to the use of cells in animals using Bio-Glo™ Luciferase Assay System according to the manufacturer's instructions (Promega, Mannheim, Germany).

2.7. Bioluminescence Imaging

In vivo bioluminescence was imaged with an IVIS® Spectrum imaging system (Perkin Elmer) 5 min after *i.p.* injection of luciferin (150 mg/kg body weight (BW)). Imaging data were analyzed with Living Image® 4.1 software (Perkin Elmer, USA). Quantitative data were obtained by choosing respective regions of interest (ROI) with a diameter of 25 mm in tumor and skin region. Quantitative intensity data were based on total radiant efficiency (TRE), accounting for the sum of all signals in respective ROIs. Since tumors were growing subcutaneously, the tumor signal was calculated by subtracting the respective signal for skin from recorded TRE in the tumor region. The animals were sedated with isoflurane gas anesthetic during imaging.

2.8. Therapy with PLD

The experiments were started 10 to 14 days after cell inoculation once the tumors measured ~1 cm^3. PLD was injected *i.v.*at different doses (4.5 mg/kg BW n=3; 9 mg/kg BW n=12 and 14 mg/kg BW n=5). Tumor size was measured by caliper over time. In the plasmapheresis group, PLD plasmapheresis was performed after 24 h; 36 h or 48 h when

9 mg/kg PLD were given, or plasmapheresis was performed after 36 h when 14 mg/kg BW were given. We euthanized animals when tumor size exceeded 3 cm^3 or body weight dropped by more than 20% over 2 days during therapy.

2.9. Plasmapheresis

Discontinuous plasmapheresis (DCP) was performed as described earlier [11]. Briefly, heparin (100 IU/ kg BW) was injected *i.v.* through venous access *via* the tail vein. A 1.5 ml blood sample was taken and centrifuged (4500 g; 1 min) to separate plasma from cellular components. The plasma was aspired and filtered. Filters (10 mm diameter x 60 mm length) were custom-made by Membrana GmbH (Wuppertal, Germany), containing polyethersulfone hollow fibers Fraktio-PES® 40/200 (inner surface area 5.8 cm^2). The filtrate and cellular components were reunited and returned to the rat *via* the venous blood access. This step was repeated up to 4 times. Plasmapheresis took ~1h while the animals were kept under isoflurane anesthesia.

2.10. Toxicities

Animals were observed twice daily for toxicities. The most important dose-limiting toxicities of PLD in humans are skin toxicity (palmar-plantar erythrodysesthesia/hand-foot syndrome and mucositis), and neutropenia [9]. Blood toxicities are exemplified in the supplement (Fig. **S3**), but are biased by blood loss during plasmapheresis. Skin toxicities are observed in animals, too. Besides palmar-plantar erythrodysesthesia along the animals' paws, skin toxicity emerges as hair loss, erythema and impairment around the eyes and the anal-genital region, usually accompanied by orange spots of doxorubicin. The toxicities we observed were classified into four categories: (+) mild - observed only on close inspection (fewer than 4 spots or total surface area < 0.5 mm^2); (++) medium- easily observed (more than 3 spots or total surface area 0.5 to 2 mm^2); (+++) severe - significantly hampering animal health (eg, hampered motility, food intake, visibility), (++++) very severe, necessitating euthanasia. We relied on animal weight as a surrogate parameter for overall toxicity, and weight loss of >20% during therapy lead to euthanasia according to animal welfare regulations.

2.11. Data Handling

Data plots and curve fits were created using Graph Pad Prism software version 7.0 (La Jolla California, USA). Variance is given either as 95% confidence interval (95%CI) or s.d. (Gaussian distribution tested by Shapiro-Wilk criteria with p=0.05).

3. RESULTS

3.1. Liposomes

The PLD used (Doxoves®) had essentially the same composition and size distribution as Doxil®/Caelyx® used in clinical practice. Average doxorubicin concentration was 4.0 (± 0.1) mg/ml, lipid concentration was 40 (± 0.1) mg/ml, and median size was 89.3 (± 0.7) nm with a PDI of 0.03 (± 0.01). For more details, see the certificate of analysis as stated by the manufacturer (http://www.liposomeexpert.com/categories/Drug-Loaded-Liposomes/). PLD elimination in plasma could be described by first-order kinetics as previously reported [17]. With the Fisher rats used, the plasma half-life of PLD depended on the PLD plasma concentration (Fig. **1**). When 4.5 mg PLD/kg BW were injected *i.v.*, plasma half-life was ~35 h (95%CI 31-40 h), increasing to 52 h (95%CI 47-54 h) or 73 h (95%CI 63-90 h) when 9 mg/kg BW or 14 mg/kg BW were used.

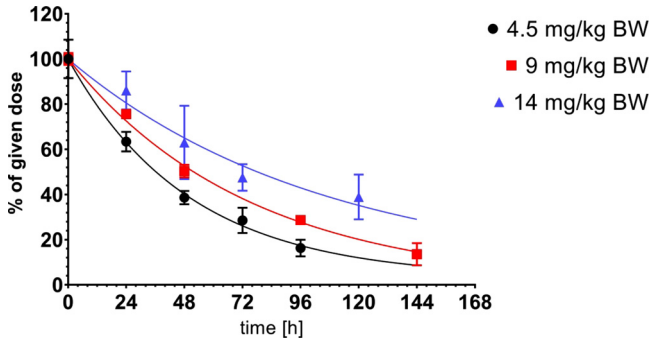

Fig. (1). Plasma elimination of PLD. Animals were injected i.v. with PLD at different concentrations. Plasma concentration right after injection was set as 100%, and elimination was fitted by first order kinetics (n=6).

3.2. Plasmapheresis

To eliminate circulating PLD, discontinuous plasmapheresis was used as previously described [11]. Separated plasma was filtered *via* a custom-made hollow fiber module. As already seen with plasmapheresis procedures used in clinical practice [18], neither liposomes nor free doxorubicin was detected in the filtrate (supplement Fig. **S1**). For the experiments with tumor-bearing animals, we took 4 wash steps, leading to an average reduction in the plasma doxorubicin concentration of 45% (± 9%), as expected [11]. The plasma pool of a 200 g rat was roughly estimated to 5 ml. Simulating discontinuous plasmapheresis with 4 wash steps in a plasma pool of 5 ml eliminated approx. 50% of albumin, lipoproteins or immunoglobulins (see supplement Fig. **S2**). The retention of these plasma components was more pronounced than that observed with clinical used double filtration plasmapheresis [13]. Thus the simplified plasmapheresis procedure used within this experimental setting is well-suited to reflect a clinical approach, but simplified plasmapheresis inflicts a greater burden on the animals than more advanced plasmapheresis techniques do on human patients.

3.3. Bioluminescence

To follow living tumor cells by *in vivo*-imaging, MAT-B-III cells were transfected with a luciferase reporter plasmid. Luciferin was injected *i.p.* and bioluminescence was measured 5 min after injection. Tumor regions exhibited significantly more bioluminescence intensity than normal skin regions (Fig. **2**). Over time, the bioluminescent signal failed to correlate with traditional tumor size measurements (Fig. **3**). For 4.5 mg PLD/kg BW, both the tumor size and bioluminescence intensity increased within the first days. Unfortunately, the bioluminescent signal weakened after 4 days although the tumor was still growing. At dosages of 9 mg doxorubicin/kg BW, both tumor size and the bioluminescent signal lessened during therapy, with the latter decreasing

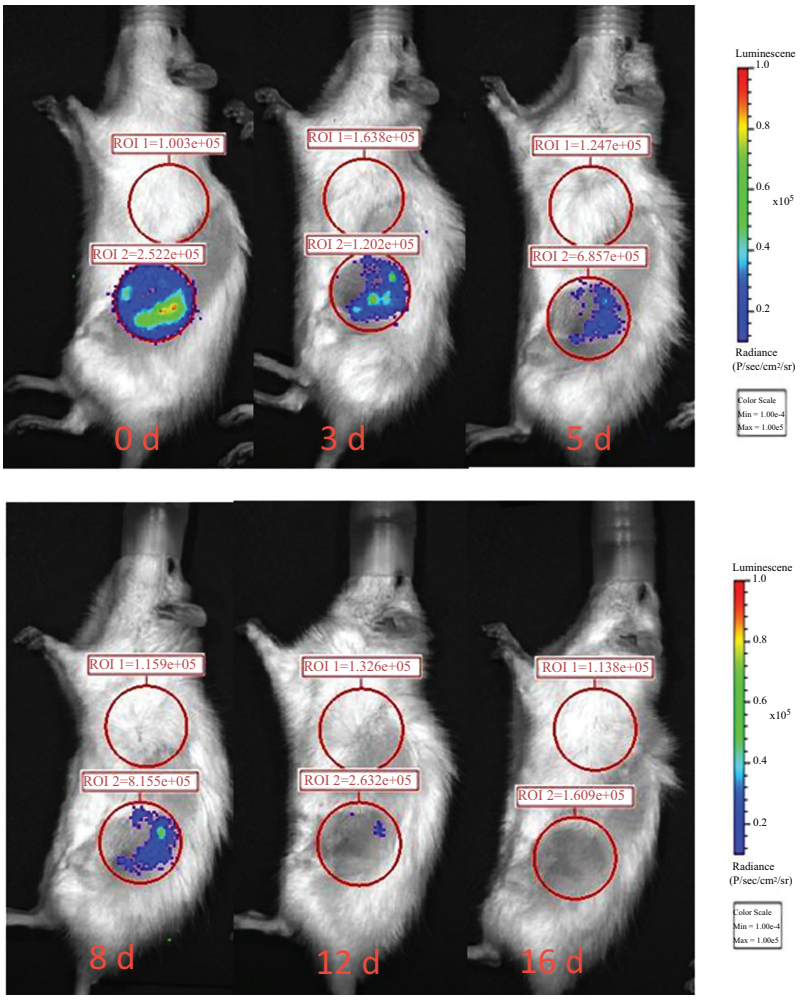

Fig. (2). Bioluminescence imaging of tumor cell viability. Female Fisher rats were inoculated with MAT-B-III tumor cells transfected with a luciferase reporter plasmid. Once tumors measuring 0.5 - 1 cm^3 had developed, therapy with PLD was initiated (day 0). Bioluminescence was measured immediately after injection of 150 mg/kg BW luciferin i.p. Maximum intensities were recorded. Arbitrary color scale was between $1.0x10^4$ and $1.0x10^5$ [p/s/cm^2/sr]. A typical example of therapy with PLD 9 mg/kg BW observed for 16 days is illustrated.

more rapidly. As with 9 mg/kg BW, at dosages of 14 mg PLD/kg BW we observed a sharp drop in bioluminescence within the first days, indicating a therapeutic impact on cell viability. In contrast, tumor size did not decrease within the observation period. Since rats had to be euthanized due to significant weight loss, we could not monitor tumor development over a longer period, and we cannot conclude whether tumor size or bioluminescence reflects the therapeutic impact more accurately. Taken together, bioluminescence may be useful to demonstrate the therapeutic impact on tumor cell viability, but the decrease in cell viability does not correlate well with tumor size in the animal model we used. Although the bioluminescence decreases, tumors may still grow. Thus bioluminescence may complement tumor size measurements, but replacing traditional tumor volume measurements by bioluminescence techniques should be considered with great caution.

3.4. Therapy with PLD

We used different dosages of PLD to investigate the impact of scheduled plasmapheresis on therapeutic efficacy and adverse events. Control animals did not undergo plasmapheresis. PLD at a dose of 4.5 mg did not suffice to achieve tumor regression (Fig. **3**), thus that dosage was not investigated further. When 9 mg PLD/kg BW were injected as a single bolus, we observed a significant but slow reduction in tumor volume over 25 days in the control group (Fig. **4A**). Average tumor volume increased when plasmapheresis was performed 24 h after the PLD injection. When plasmapheresis was carried out 36 h after the PLD injection, initial growth within the first days was followed by significant tumor shrinkage. By the end of the observation period, average tumor size was similar to the control group's tumor size. When plasmapheresis was performed after 48 h, tumor size decreased faster than in the control group (Fig. **4C**), but we could not follow those animals any longer due to pronounced weight loss.

To monitor the animals' general well-being, their weight was recorded (Figs. **4B**/**D**). All animals lost weight after the PLD injection, indicating the impact of chemotherapy on general health. However, those that underwent plasmapheresis after 24 or 36 h regained their initial weight much faster than the control animals. When plasmapheresis was done after 48 h, the weight loss was more pronounced, and ani-

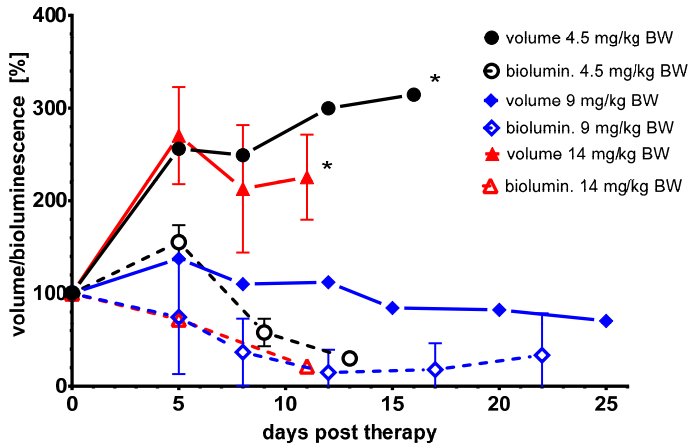

Fig. (3). Correlation between tumor volume and bioluminescence intensity. Female Fisher rats were inoculated with MAT-B-III cells that were transfected with a luciferase reporter plasmid. Once tumors had grown, they were treated with different concentrations of PLD (4.5 mg/kg BW, black line (n=3); 9 mg/kg BW blue line (n=12); 14 mg/kg BW (n=5). Tumor volume (solid symbols, solid line) and bioluminescence (open symbols, dashed lines) were measured; * indicates that rats had to be euthanized. *(The color version of the figure is available in the electronic copy of the article).*

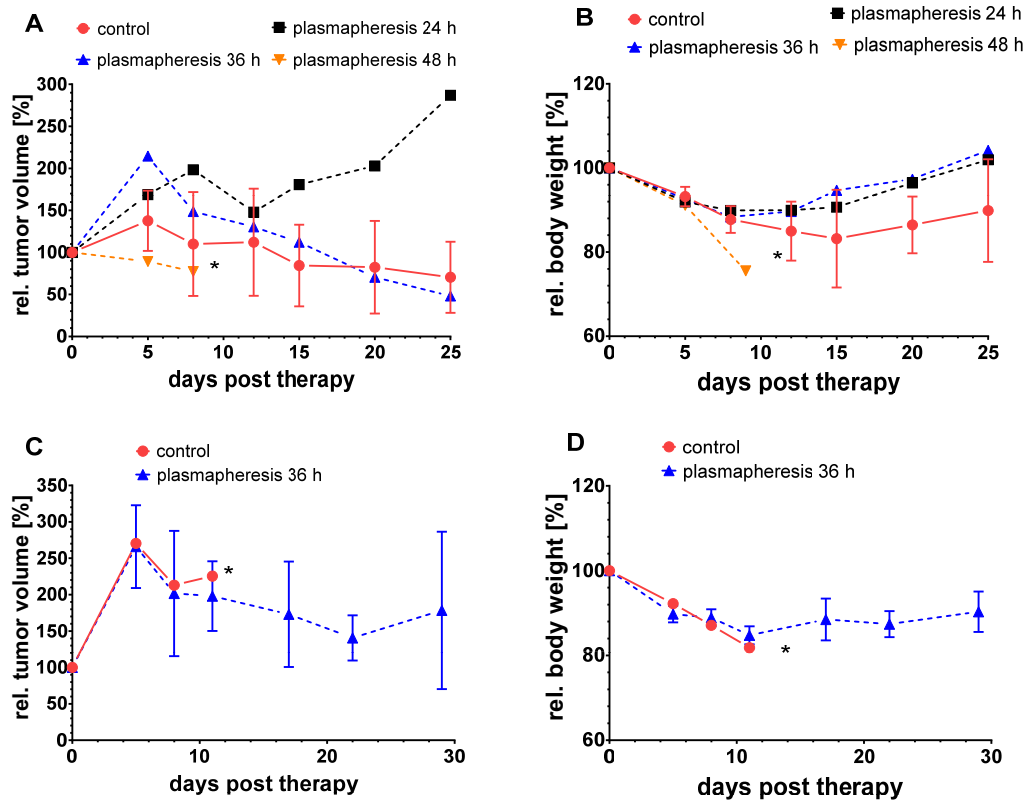

Fig. (4). Treatment of tumor-bearing rats with PLD. Syngeneic tumors (MAT-B-III) were grown in female Fisher rats to measure 0.5-1.0 cm^3. Tumor size and body weight at start of treatment were defined as 100%. A,B: PLD 9 mg/kg BW, control red line (n=12), plasmapheresis after 24 h:black line (n=7); 36 h: blue line (n=8) or 48 h: orange line (n=3); C,D: 14 mg/kg BW control red line (n=5); 36 h: blue line (n=7); A,C: tumor size development; B,D body weight development. * indicates when rats were euthanized because of body weight loss >20%. *(The color version of the figure is available in the electronic copy of the article).*

mals had to be euthanized according to animal welfare regulations. These data indicate that discontinuous plasmapheresis of PLD stresses the animals to a certain extent. The weight loss is probably related to the pronounced retention of plasma components (Fig. **S2**) and loss of blood cells (Fig. **S3**). When plasmapheresis is done early, the benefits of reduced PLD toxicity outweigh the additional burden, but when done too late, the adverse effects outweigh the benefits. When plasmapheresis is timed appropriately, animal health is improved while maintaining antitumor efficacy.

Interestingly, raising the PLD dosage to 14 mg doxorubicin/kg BW did not lead to greater antitumor efficacy (Fig. **3**) than a dosage of 9 mg/kg BW. Control animals had to be euthanized because of weight loss (Fig. **4D**). When plas-

mapheresis was done after 36 h, animal weight stabilized. After a brief initial growth phase, tumor size decreased for more than 20 days. Not surprisingly, the animals' weight loss was more pronounced in conjunction with a higher dosage. As with 9 mg PLD/kg BW, plasmapheresis after 36 h both benefited the animals and demonstrated significant antitumor efficacy. When dosing 14 mg PLD/kg BW, we refrained from other plasmapheresis schedules for animal-welfare reasons.

3.5. Toxicities

In addition to animal weight, we documented the most common adverse events associated with the treatment (Table **1**). These were PPE and erythema, hair loss and diminished hair regrowth in the regions that had been shaved, as well as disorders in the anal-genital region and around the eyes (see 2.10 for details). Fig. (**5**) illustrates some typical examples. Besides severe weight loss, we observed no grade 4 toxicities. Overall, side effects were more common and more severe in the control group than in the respective plasmapheresis groups, indicating a significant benefit from plasmapheresis on the toxicity profile (supplement tab. S1). The side effects' severity and frequency increased with the delay prior to plasmapheresis. This finding highlights the relationship between adverse effects and the AUC of PLD. Higher dosages are of course associated with more severe side effect profiles, but again plasmapheresis was beneficial.

4. DISCUSSION

4.1. Liposomes

Commercial PLD is characterized by very long plasma half-life and low distribution volumes conferring mostly to the plasma compartment [17]. The plasma half-lives we noted were in the range previously observed in conjunction with PLD. In our rat model, we observed a certain but nonlinear increase in PLD plasma half-life with increasing dosage, as observed before for long-circulating liposomes in mice as well [19].

4.2. Bioluminescence Imaging

The impact of therapy on tumor development is usually measured by monitoring tumor size. Accurate three-dimensional measurements of tumor size are often difficult to take even when the tumor grows subcutaneously. Tumor geometry is usually somewhat irregular, and tumor penetration into tissue is even more difficult to assess. Alternatively, bioluminescence imaging is a noninvasive technique that may provide data on tumor cell viability and growth [15]. Luciferase expression and bioluminescence does not affect tumor cell growth [20]. Unfortunately, our bioluminescent intensities measured with a luciferase reporter plasmid did not correlate well with tumor size measurements in the rat tumor model we used. While bioluminescence seems more sensitive to reveal an initial chemotherapeutic impact, tumors continued to grow despite a significant reduction in the bioluminescence signal. While bioluminescence imaging has proven very useful in detecting the early growth of small tumors and metastases, there is also evidence of the growth of large tumors without an increasing bioluminescence signal [21, 22]. On the other hand, tumors may contain sclerotic and necrotic regions that contribute to their size but not to further development, and killing a fraction of active tumor cells is not necessarily reflected in shrinkage of tumor size. Because of the lack of selection advantage for plasmid expression *in vivo*, loss of reporter plasmid may be another limitation of the bioluminescence method used, and more stable genetic models may lead to better results. So far, bioluminescence may be used to provide additional information on the early impact of a therapy on cell viability, but it cannot replace tumor size measurements *via* conventional methods in the long run. To minimize the animals' stress level, we monitored therapeutic efficacy in the plasmapheresis groups only by measuring the tumor size in the plasmapheresis experiments.

4.3. Plasmapheresis and the Balance between Efficacy and Adverse Events

Chemotherapy dosing is always a difficult balancing act between toxicity against tumor cells and toxicity to healthy

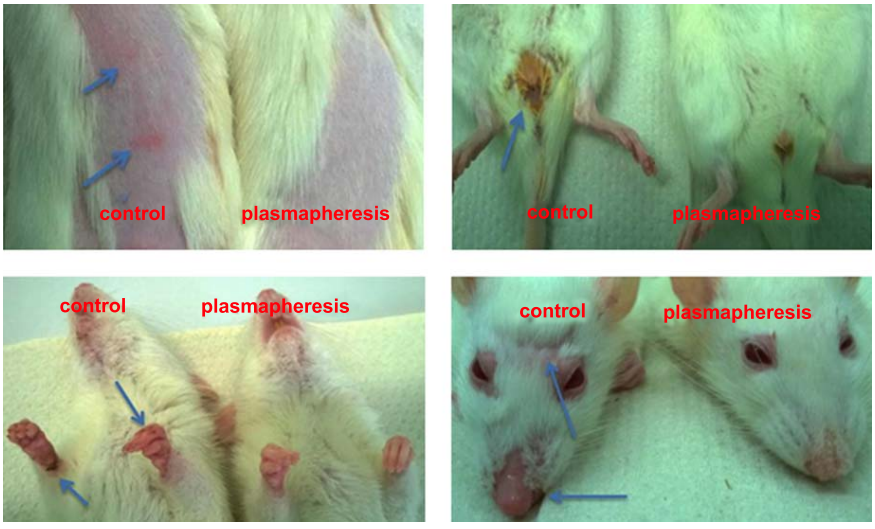

Fig. (5). Typical PLD toxicities. Female Fisher rats were injected with PLD (9 or 14 mg/kg BW, see Fig. **4**) for tumor treatment. **A**) skin toxicities; **B**) disorders in the anal-genital region; **C**) palmar-plantar erythrodysesthesia (PPE); **D**) hair loss.

Table 1. Adverse events during PLD treatment. The numbers of animals exhibiting adverse events are given for each treatment group. Adverse events were classified as absent (0); mild (+); severe (++) and very severe (+++). Mild side effects were those only detected by close inspection, severe adverse events were easily spotted, very severe adverse events did lead to significant animal impairment, and worsening to euthanasia (see 2.10 for details).

Dosing		**9 mg/KG BW**				**14 mg/kg BW**	
Plasmapheresis		**no**	**24h**	**36h**	**48h**	**no**	**36h**
Toxicity	grade						
PPE	0	-	5	5	1	-	-
	+	-	2	3	1	-	-
	++	12	-	-	1	-	7
	+++	-	-	-	-	5	-
erythema	0	-	7	4	-	-	2
	+	-	-	3	-	-	-
	++	12	-	1	3	-	5
	+++	-	-	-	-	5	-
eyes	0	-	7	6	2	-	-
	+	-	-	2	-	-	-
	++	12	-	-	1	-	7
	+++	-	-	-	-	5	-
anal/genital	0	-	7	6	-	-	-
	+	12	-	2	3	-	-
	++	-	-	-	-	-	7
	+++	-	-	-	-	5	-
hair growth	0	-	4	6	1	-	-
	+	12	3	2	2	5	7
animals observed		*12*	*7*	*8*	*3*	*5*	*7*

tissues. A dosage of 4.5 mg PLD/kg BW revealed no obvious adverse reactions, but failed to trigger significant tumor regression. Increasing the dosage to 9 mg/kg BW led to significant tumor regression. Concurrently toxicity increased, and grade 2 adverse events were observed in all control group animals. We recently demonstrated that scheduled plasmapheresis does not affect the accumulated maximum amount of liposomes in tumors, but it does reduce the amount of accumulated liposomes in several tissues like skin and paws, where major adverse reactions to PLD occur [11]. Within these experiments, plasmapheresis was performed after 24 h, which was slightly less than the half-life of the fluorescent liposomes used. After plasmapheresis, skin fluorescence dropped between 24 and 48 h, while the tumor's peak concentration remained unchanged. In a therapeutic setting, plasmapheresis may be best timed when the difference is maximum between tumor accumulation and the accumulation in tissues affected by major side effects. On the other hand, plasmapheresis will only be effective when significant amounts of liposomes are eliminated. With plasmapheresis eliminating ~45% of circulating liposomes, we assumed a minimum of 60% of initial dosage circulating in the plasma to be efficient. In the animal model we used, PLD had a plasma-half of ~53 h (9mg/kg BW). Therefore we investigated plasmapheresis after 48 h, and subsequently plasmapheresis timed at 36 and 24 h.

When PLD was given at 9 mg/kg BW, and plasmapheresis was performed after 48 h, antitumor efficacy was similar to the control groups. This is in line with accumulation data studied with fluorescent long-circulating liposomes, where we observed a maximum accumulation around a flat plateau phase between ~ 48-90 h [11], which is in line with findings in other animal models [23, 24]. PLD specific toxicities like skin toxicities significantly decreased in the plasmapheresis group. Due to chemotherapy, all animals lost weight. As those in the 48-h-plasmapheresis group, unfortunately, lost more than 20% weight within the first week, they had to be euthanized due to animal welfare regulations and we could not follow them any longer. Plasmapheresis, as carried out in the small animal model we used, is not as highly sophisti-

cated as the double filtration plasmapheresis process used in clinical practice [18]. Loss of blood cells and prominent loss of plasma proteins cause an additional unspecific burden for the animals that is reflected in their blood parameters and likely in initial weight loss. Taken together, no net benefit was gained when plasmapheresis was performed 48 h after PLD injection despite a certain reduction in PLD-specific toxicities.

When plasmapheresis took place 36 h after dosing, the antitumor efficacy was similar to the control groups. With fluorescent liposomes, we observed ~95% of maximum accumulation after 36 h [11], which seems to be sufficient to maintain an effective antitumor response. PLD-specific adverse events were significantly fewer in the plasmapheresis group than in the control animals (see tab 1 and supplemental tab S1). Skin toxicities are a major dose-limiting toxicity for PLD in humans, and the reduction in skin toxicities by *via* plasmapheresis that we observed in our animals is in line with diminished skin toxicities in humans [13]. While their initial weight loss was similar to the control group's, the animals in the plasmapheresis group recovered faster than the control animals over time. Despite a certain degree of blood loss and loss of plasma proteins, there is an overall net benefit by eliminating circulating PLD after 36 h. Note that we observed a non-significant tendency toward a less effective antitumor response during the first days, but therapy became even more effective over time. This may reflect the complex balancing act between the tumor, the host's antitumor response and chemotherapy, which seems well worth elucidating in more detail in further experiments, using immunocompetent animal models.

When plasmapheresis was performed after 24 h, adverse events were very mild or absent, but antitumor efficacy was also hampered. Obviously, tumor accumulation has not proceeded to the extent necessary for sufficient response, which concurs with previous accumulation data, where ~75% of maximum tumor accumulation was achieved after 24 h [11]. Taken together, the timing of plasmapheresis is crucial to achieve sufficient antitumor response in conjunction with diminished toxicities. When timing is appropriate, plasmapheresis of PLD can reduce toxicities while maintaining antitumor efficacy.

Nanoparticles accumulate in solid tumors primarily *via* the EPR-effect, and accumulation is linearly proportional to the dosage [11, 25, 26]. Interestingly, raising the dosage to 14 mg/kg BW did not trigger a better antitumor response, while toxicities worsened as expected. Using a syngeneic immune-competent animal model, increasing the dosage may impair the host's antitumor defense, thereby undermining a presumably more effective eradication of tumor cells**.** Inhomogeneous distribution within tumor tissue [27] may be another reason for the observed effect. Increasing the dosage to a "local overkill" does not help to eradicate tumor cells in parts of the tumor where no accumulation occurs. Combining 14 mg PLD/kg BW and plasmapheresis after 36 h, we noted a significant reduction in toxicities, and animals could be followed for a longer time. A sustained tumor static response was observed, but the overall efficacy was less than that associated with 9 mg PLD/ kg BW.

The impact of nanomedicine on current cancer therapy remains far behind its potential [28]. Making a point of reducing toxicities while developing DDS-based therapies may help to better harvest the potential of nanomedicine. Discontinuous plasmapheresis and double filtration plasmapheresis are generally suitable for many nanoparticles and nanostructures of appropriate size, regardless of their composition [29]. As schematically highlighted in Fig. (**6**), this clinically approved technology offers the unique possibility to shift the balance between efficacy and toxicity for various DDS in anticancer therapy.

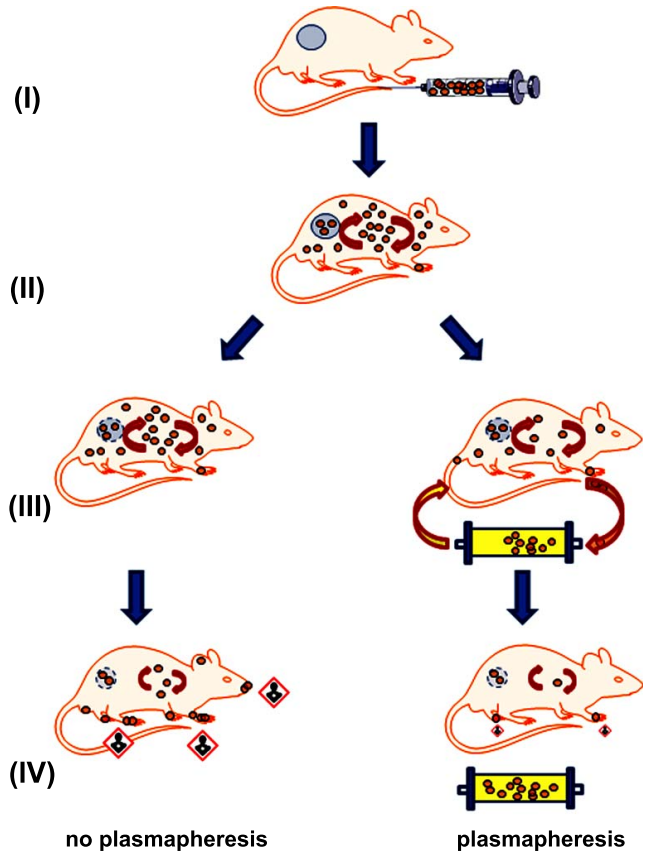

Fig. (6). Plasmapheresis to diminish toxicities of nanoparticle based antitumor therapy. Nanoparticle based drug delivery systems (DDS) are injected into tumor bearing mice (I). Accumulation of DDS in tumor by EPR-effect over several hours is faster than accumulation in non-target tissues (II). Once accumulation has peaked, excess of circulating DDS is eliminated by plasmapheresis (III). Without plasmapheresis, excess of DDS accumulate in non-target tissue, leading to severe toxicities. With plasmapheresis, accumulation in non-target tissue is reduced and toxicities are diminished (IV). DDS: red circles; tumor: blue spot.
This llustration was created with the help of Servier Medical Art repository (https://smart.servier.com/) under Creative Commons Attribution 3.0 Unported License. *(The color version of the figure is available in the electronic copy of the article).*

CONCLUSION

Toxicities and therapeutic success are critically intertwined since chemotherapy affects both tumor growth and the body's natural defense mechanisms. Attacking the tumor with higher doses is not necessarily more successful when natural defense mechanisms are impaired. Eliminating the

(necessary) excess of circulating nanoparticles enables an efficient therapy in conjunction with reduced toxicities when plasmapheresis is carried out at the right time-point.

LIST OF ABBREVIATIONS

BW = Body Weight
DCP = Discontinuous Plasmapheresis
DDS = Drug Delivery Systems
EPR = Enhanced Permeation And Retention
HSPC = Hydrogenated Soy Phosphatidylcholine
PDI = Polydispersion Index
PLD = Pegylated Liposomal Doxorubicin
ROI = Regions Of Interest
TRE = Total Radiant Efficiency

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Regierungspräsidium Freiburg; Referat 35; Veterinärwesen, Freiburg, Germany (reference G07/60 and G12/09).

HUMAN AND ANIMAL RIGHTS

No Humans were used for studies that are the basis of this research. Animal experiments were performed according to national and international guidelines (EU Directive 2010/63/EU).

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author Gerhard Pütz holds a patent on the extracorporeal elimination of potentially toxic or harmful substances (EP20020759794).

The research was financed by the Faculty of Medicine of the University Freiburg Medical Center, IVIS scanner and respective staff was financed by the Deutsche Forschungsgemeinschaft DFG (SFB850).

ACKNOWLEDGEMENTS

The authors are very grateful to Vitalij Maks for his extraordinarily skillful animal handling. Size measurements were taken with kind assistance from Prof. U. Massing, Hettich GmbH, Freiburg, Germany. This research was financed by the Faculty of Medicine of the University Freiburg Medical Center, IVIS scanner and the respective staff was financed by the Deutsche Forschungsgemeinschaft DFG (SFB850).

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

[1] Shi, J.; Kantoff, P.W.; Wooster, R.; Farokhzad, O.C. Cancer nanomedicine: Progress, challenges and opportunities. *Nat. Rev. Cancer,* **2017**, *17*(1), 20-37.

[2] Gregoriadis, G.; Ryman, B.E. Fate of Protein-containing liposomes injected into rats: An approach to the treatment of storage diseases. *Eur. J. Biochem.,* **1972**, *24*(3), 485-491.

[3] Olov, N.; Bagheri-Khoulenjani, S.; Mirzadeh, H. Combinational drug delivery using nanocarriers for breast cancer treatments: A review. *J. Biomed. Mater. Res. A,* **2018**.

[4] Komiyama, M.; Yoshimoto, K.; Sisido, M.; Ariga, K. Chemistry can make strict and fuzzy controls for bio-systems: DNA nanoarchitectonics and cell-macromolecular nanoarchitectonics. *Bull. Chem. Soc. Jpn.,* **2017**, *90*(9), 967-1004.

[5] Ramasamy, T.; Ruttala, H.B.; Gupta, B.; Poudel, B.K.; Choi, H.G.; Yong, C.S.; Kim, J.O. Smart chemistry-based nanosized drug delivery systems for systemic applications: A comprehensive review. *J. Control. Release,* **2017**, *258*, 226-253.

[6] Matsumura, Y.; Maeda, H. A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res.,* **1986**, *46*(12 Part 1), 6387-6392.

[7] Maeda, H. Macromolecular therapeutics in cancer treatment: The EPR effect and beyond. *J. Control. Release,* **2012**, *164*(2), 138-144.

[8] Barenholz, Y. (Chezy). Doxil® - the first FDA-approved nano-drug: Lessons learned. *J. Control. Release,* **2012**, *160*(2), 117-134.

[9] Gabizon, A.; Shmeeda, H.; Grenader, T. Pharmacological basis of pegylated liposomal doxorubicin: Impact on cancer therapy. *Eur. J. Pharm. Sci.,* **2012**, *45*(4), 388-398.

[10] Pütz, G.; Schmah, O.; Eckes, J.; Hug, M.J.; Winkler, K. Controlled application and scheduled removal of nanoparticle based chemotherapeutics (CARL) will reduce dose limiting adverse events in anticancer chemotherapy. *Med. Hypotheses,* **2009**, *72*(4), 393-397.

[11] Ngoune, R.; Peters, A.; von Elverfeldt, D.; Winkler, K.; Pütz, G. Accumulating nanoparticles by EPR: A route of no return. *J. Control. Release,* **2016**, *238*, 58-70.

[12] Bambauer, R.; Bambauer, C.; Lehmann, B.; Latza, R.; Schiel, R. LDL-Apheresis: Technical and clinical aspects. *Sci. World J.,* **2012**, *2012*, 314283-314283.

[13] Eckes, J.; Schmah, O.; Siebers, J.W.; Groh, U.; Zschiedrich, S.; Rautenberg, B.; Hasenburg, A.; Jansen, M.; Hug, M.J.; Winkler, K.; Pütz, G. Kinetic targeting of pegylated liposomal doxorubicin: A new approach to reduce toxicity during chemotherapy (CARL-Trial). *BMC Cancer,* **2011**, *11*, 337.

[14] Martínková, J.; Bláha, M.; Kubeček, O.; Maláková, J.; Špaček, J.; Bezouška, J.; Krulichová, I.S.; Filip, S. Plasmafiltration as a possible contributor to kinetic targeting of pegylated liposomal doxorubicin (PLD) in order to prevent organ toxicity and immunosuppression. *Cancer Chemother. Pharmacol.,* **2016**, *77*(2), 429-437.

[15] O'Farrell, A.; Shnyder, S.; Marston, G.; Coletta, P.; Gill, J. Non-invasive molecular imaging for preclinical cancer therapeutic development. *Br. J. Pharmacol.,* **2013**, *169*(4), 719-735.

[16] Stewart, J.C.M. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal. Biochem.,* **1980**, *104*(1), 10-14.

[17] Gabizon, A.; Shmeeda, H.; Barenholz, Y. Pharmacokinetics of pegylated liposomal doxorubicin. *Clin. Pharmacokinet.,* **2012**, *42*(5), 419-436.

[18] Pütz, G.; Schmah, O.; Eckes, J.; Hug, M. J.; Winkler, K. Controlled application and removal of liposomal therapeutics: Effective elimination of pegylated liposomal doxorubicin by double-filtration plasmapheresis *in vitro*. *J. Clin. Apheresis* **2010**, *25*(2), 54-62.

[19] Allen, T.M.; Hansen, C. Pharmacokinetics of stealth versus conventional liposomes: Effect of dose. *Biochim. Biophys. Acta BBA - Biomembr.,* **1991**, *1068*(2), 133-141.

[20] Tiffen, J.C.; Bailey, C.G.; Ng, C.; Rasko, J.E.; Holst, J. Luciferase expression and bioluminescence does not affect tumor cell growth *in vitro* or *in vivo*. *Mol. Cancer,* **2010**, *9*, 299.

[21] Jurczok, A.; Fornara, P.; Söling, A. Bioluminescence imaging to monitor bladder cancer cell adhesion *in vivo*: A new approach to optimize a syngeneic, orthotopic, murine bladder cancer model. *BJU Int.,* **2008**, *101*(1), 120-124.

[22] Dickson, P.V.; Hamner, B.; Ng, C.Y.C.; Hall, M.M.; Zhou, J.; Hargrove, P.W.; McCarville, M.B.; Davidoff, A.M. *In vivo* bioluminescence imaging for early detection and monitoring of disease progression in a murine model of neuroblastoma. *J. Pediatr. Surg.,* **2007**, *42*(7), 1172-1179.

[23] Hagtvet, E.; Evjen, T.J.; Nilssen, E.A.; Olsen, D.R. Assessment of liposome biodistribution by non-invasive optical Imaging: A feasibility study in tumour-bearing mice. *J. Nanosci. Nanotechnol.,* **2012**, *12*(3), 2912-2918.

[24] Maeda, H. Toward a full understanding of the epr effect in primary and metastatic tumors as well as issues related to its heterogeneity. *Adv. Drug Deliv. Rev.,* **2015**, *91*, 3-6.

[25] Northfelt, D.W.; Martin, F.J.; Working, P.; Volberding, P.A.; Russell, J.; Newman, M.; Amantea, M.A.; Kaplan, L.D. Doxorubicin encapsulated in liposomes containing surface-bound polyethylene glycol: Pharmacokinetics, tumor localization, and safety in patients with AIDS-related kaposi's sarcoma. *J. Clin. Pharmacol.,* **1996**, *36*(1), 55-63.

[26] Charrois, G.J.R.; Allen, T.M. Multiple injections of pegylated liposomal doxorubicin: Pharmacokinetics and therapeutic activity. *J. Pharmacol. Exp. Ther.,* **2003**, *306*(3), 1058-1067.

[27] Stapleton, S.; Milosevic, M.; Tannock, I.F.; Allen, C.; Jaffray, D. A. The intra-tumoral relationship between microcirculation, interstitial fluid pressure and liposome accumulation. *J. Control. Release*., **2015**, *211*, 163-170.

[28] Venditto, V.J.; Szoka Jr., F.C. Cancer nanomedicines: So many papers and so few drugs. *Adv. Drug Deliv. Rev.,* **2013**, *65*(1), 80-88.

[29] Pütz, G.; Eckes, J.; Schmah, O.; Winkler, K.; Wieland, H. Elimination of liposomes by different separation principles used in low-density lipoprotein apheresis. *Ther. Apher. Dial.,* **2008**, *12*(1), 2-12.