International Journal of Pharmaceutics 411 (2011) 206–214

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Effective tumor targeting and enhanced anti-tumor effect of liposomes engrafted with peptides specific for tumor lymphatics and vasculature

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ARTICLE INFO

Article history:
Received 17 December 2010
Received in revised form 10 March 2011
Accepted 21 March 2011
Available online 4 April 2011

Keywords:
Stealth liposomes
Chelator lipid
Targeting peptide
Doxorubicin
Vasculature
Drug delivery

ABSTRACT

The use of liposomes to target drugs to tumors represents an attractive therapeutic strategy, especially when used with convenient targeting moieties such as peptides. Here we explored several peptides for their ability to target liposomes to tumors. The metal chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA_3-DTDA) was incorporated into liposomes to enable the engraftment of His-tagged peptides containing targeting motifs specific for tumor vasculature markers VEGFR-1 (p39-Flt-1) and neuropilin-1 (p24-NRP-1), or a motif known to accumulate in hypoxic areas of tumors (p47-LyP-1). Peptide-engrafted liposomes were examined for their biodistribution and anti-tumor effects after i.v. administration. Our results show that radiolabelled liposomes engrafted with either p24-NRP-1 or p47-LyP-1 and then injected into mice bearing subcutaneous B16-F1 tumors, show increased accumulation in the tumor. For p24-NRP-1-liposomes, tumor targeting was significantly increased when the stabilizing lipid phosphatidylethanolamine polyethylene glycol-750 (PE-PEG_{750}) was used instead of PE-PEG_{2000} in the liposome lipid mixture. Importantly, compared to the controls, p24-NRP-1 liposomes containing 10 mol% PE-PEG_{750} and loaded with doxorubicin significantly inhibited the rate of tumor growth in the tumor-bearing mice. Our findings demonstrate that the use of drug-containing liposomes incorporating NTA_3-DTDA and engrafted with NRP-1 targeting peptide is a convenient strategy to enhance the therapeutic effect of non-targeted doxorubicin.

1. Introduction

The ability to target liposomal drugs to tumors represents an attractive strategy for developing more effective cancer therapies. Adequate targeting of liposomes to tumors has been difficult to achieve, however, often due to the poor tumor penetrability of liposomes, and difficulties associated with the attachment of suitable liposome-targeting moieties. The endothelial cells that form the walls of tumor blood vessels present a significant barrier to blood-borne therapeutics, yet these cells also represent an attractive target for anti-tumor therapy (Chen et al., 2009; Satchi-Fainaro, 2002). Thus, it is reported that the tumor vasculature plays a major role in supporting tumor growth, and that destruction of tumor blood vessels can lead to tumor regression by blocking the supply of oxygen and nutrients needed for growth (Lammers et al., 2008). Endothelial cells associated with the tumor are directly accessible to systemically administered therapeutics, and are more genetically stable and therefore less prone to drug resistance compared to cells of the tumor (Molema et al., 1998). The ability to target liposomal drugs to the tumor vasculature could thus provide a powerful approach for cancer therapy.

New blood vessel growth within tumors occurs primarily through the process of angiogenesis (Janic and Arbab, 2010). A key factor promoting angiogenesis is vascular endothelial growth factor (VEGF), which can be secreted by tumor cells, and binds to receptors expressed on endothelial cells (Ferrara et al., 2003). Recently, phage display has led to the identification of the peptide WHSDMEWWYLLG as an antagonist for VEGFR-1 (Flt-1) (An et al., 2004); while in a separate study the peptide ATWLPPR was found to bind specifically to neuropilin-1, a VEGFR-2 co-receptor (Starzec et al., 2006). Interestingly, when injected into tumor-bearing mice, ATWLPPR-photosensitizer conjugates accumulate in the tumor vasculature (Thomas et al., 2008). Tumors also contain lymphatic vessels that are morphologically distinct from normal lymphatics, and a peptide LyP-1 (CGNKRTRGC) is reported to preferentially target tumor lymphatics (Laakkonen et al., 2002). LyP-1 is purported to bind the mitochondrial protein p32 (gC1qR), which is aberrantly expressed on the surface of cells in the lymphatics and hypoxic regions within tumors (Fogal et al., 2008). The LyP-1 pep-

Abbreviations: NTA_3-DTDA, 3(nitrilotriacetic-acid)-ditetradecylamine; 3NTA-Caelyx, Caelyx liposomes incorporated with NTA_3-DTDA; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine.

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doi:10.1016/j.ijpharm.2011.03.044

tide could thus also have potential for targeting anti-cancer drugs to tumors.

The clinically approved preparation of doxorubicin-loaded liposomes, Caelyx (Doxil in US), contains disteroylphosphatidylcholine, cholesterol and phosphatidylethanolamine-polyethylene glycol-2000 (PE-PEG_{2000}) (molar ratio 55:40:5). The PE-PEG_{2000} in this formulation confers an extended plasma half-life and the ability to avoid elimination by phagocytes of the reticulo-endothelial system. However, the long PEG_{2000} chains may also interfere with the interaction of liposome-anchored moieties to receptors (Hansen et al., 1995), and shorter PEG chains (e.g. as in PE-PEG_{750}) may be preferable in situations where the targeting moiety is anchored onto the liposome surface. In previous studies we showed that incorporation of the chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA_3-DTDA) into liposomes enables the engraftment of histidine-tagged targeting peptides (Faham et al., 2009; Herringson and Altin, 2009; Herringson et al., 2009). Also, we showed that the liposomal drug Caelyx, when incorporated with NTA_3-DTDA (3NTA-Caelyx) and engrafted with the tumor vasculature-specific peptide p46-RGD, exhibits increased anti-tumor efficacy compared to Caelyx engrafted with a control peptide (Herringson and Altin, 2010). In the present work we examined whether other tumor-targeting peptides can also be used to increase the therapeutic efficacy of liposomal doxorubicin. We prepared histidine-tagged peptides containing the targeting motifs WHSDMEWWYLLG (p39-Flt-1), ATWLPPR (p24-NRP-1) and CGNKRTRGC (p47-LyP-1), and engrafted these onto doxorubicin-containing liposomes, before i.v. injection to study their effects in tumor-bearing mice. The results show that doxorubicin-containing NTA_3-DTDA liposomes engrafted with targeting peptide (p24-NRP-1) is a convenient approach to enhance the therapeutic effect of non-targeted liposomal drug.

2. Materials and methods

2.1. Reagents

Caelyx is a product of Johnson & Johnson, and is marketed in Australia by Schering-Plough Pty Ltd. (Baulkham Hills, NSW, Australia). The chelator lipid NTA_3-DTDA was produced in the Research School of Chemistry (ANU), as described previously (Altin et al., 2006). 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)-2000] (PE-PEG_{2000}) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)-750] (PE-PEG_{750}) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). [4-^{14}C]-Cholesterol was purchased from Perkin-Elmer (Glen Waverley, VIC, Australia). The fluorescent lipid Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Texas Red-DHPE) was purchased from Molecular Probes (Eugene, OR, USA). Hoescht 33342 was kindly provided by Tom Karagiannis (Peter MacCallum Cancer Centre, Melbourne, Australia). Doxorubicin (Adriamycin) was obtained from Pfizer Australia (West Ryde, NSW, Australia). RPMI 1640 medium was obtained from the Media Unit, The John Curtin School of Medical Research (JCSMR, ANU). $NiSO_4$ was used for all of the additions of Ni^{2+} to liposome preparations. All experiments were performed using analytical grade reagents.

2.2. Cell lines and mice

The murine melanoma cell line B16-F1 was obtained from Professor Chris Parish (Department of Immunology, JCSMR, ANU). Cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), 5% newborn bovine serum, 50 μM β-mercaptoethanol, 0.1 mM L-glutamine and 0.1% PSN antibiotics solution (Media Unit, JCSMR, ANU) in a 37 °C incubator with a 5% CO_2 atmosphere. Female C57BL/6 mice were obtained from the Animal Resource Facility (Perth, WA, Australia) and were used in experiments at 8–9 weeks of age. All animal experiments were carried out in accordance with protocols approved by the Australian National University Animal Experimentation Ethics Committee.

2.3. Synthetic peptides

The following peptides were synthesized for engrafting onto NTA_3-DTDA-containing liposomes: p24-NRP-1 (GATWLPPRG-GASGASGASGASGAS-HHGHHGHHGHHGHH), p39-Flt-1 (HHHH-HHHHHHHH-GSGSGSGSGSGSGS-GWSDMEWWYLLG), p46-RGD (HHHHHHHHHHHH-GSGSGSGSGSGSGSGSGSGS-GARYCRGDCF-DG), p47-LyP-1 (HHHHHHHHHHHH-GSGSGSGSGSGSGSGSGSGS-CGNKRTRGC). A non-targeted peptide designated p49-Control (HHHHHHHHHHHH-GSGSGSGSGSGSGSGSGSGS) was used as a control to reduce nonspecific binding of NTA_3-DTDA-containing liposomes to cells. In contrast to the other targeting peptides described in this work, peptide p24-NRP-1 was produced with the His-tag at the carboxy-terminal end of the peptide to maintain the same anchoring orientation as used previously (Janssen et al., 2003). Also, p24-NRP-1 was produced in a different batch and the spacer/linker region contained GAS repeats (instead of GS repeats) and the His-tag contained four HHG repeats; these latter changes were made to facilitate synthesis and were not considered to significantly affect the activity of either the NRP-1 targeting moiety or the His-tag. All peptides were synthesized and HPLC purified by the Biomolecular Resource Facility (JCSMR, ANU). The peptides p46-RGD and p47-LyP-1 were cyclised through disulphide bond formation between the two cysteine residues. Stock solutions of peptides were made in distilled water and stored at −20 °C.

2.4. Preparation of peptide-engrafted 3NTA-Caelyx

Liposomal Caelyx stock (2 mg/mL doxorubicin) was subjected to buffer-exchange by loading onto a NICK column containing Sephadex G-50 (Amersham Biosciences AB, Uppsala, Sweden) equilibrated in PBS and eluting in PBS. Ni-NTA_3-DTDA was added (1 mol% total lipid) and incorporated with the Caelyx by incubating at 37 °C for 2 h. For engraftment with targeting peptide, the indicated histidine-tagged peptide dissolved in H_2O was added to the 3NTA-Caelyx and incubated for 30 min at room temperature as described previously for engrafting peptides to liposomes containing NTA_3-DTDA (Faham et al., 2009). The amount of peptide used for engraftment was based on a theoretical calculation of the amount of peptide required to be equimolar with anchoring moiety NTA_3-DTDA, and an empirical titration to give optimal binding of the engrafted Caelyx to target cells *in vitro* (Herringson and Altin, 2010).

2.5. Measurement of liposome-associated doxorubicin

To determine the amount of doxorubicin contained in liposomes, samples of the liposomal drug were subjected to size-exclusion chromatography by loading onto a NICK column containing Sephadex G-50 (Amersham Biosciences AB) equilibrated in phosphate buffered saline (PBS) [containing 140 mM sodium chloride and 9.5 mM sodium phosphate (pH 7.4)], hereafter referred to as PBS, and eluting in PBS. After the first 400 μL wash, liposomes containing entrapped doxorubicin were eluted in the following 400 μL while free doxorubicin was eluted in a subsequent volume of 3 mL. The doxorubicin concentration in the eluant was determined by the measurement of the absorbance at 485 nm

and comparing the reading to that from a standard curve of free doxorubicin.

2.6. Particle size measurements

The particle size of 3NTA-Caelyx liposomes was measured with a Zetasizer Nano SZ (Malvern Instruments, Worchestershire, UK). Liposomes were diluted 25 times to 500 μL in filtered PBS (pH 6.0) and transferred to a disposable 1 mL cuvette for analysis. *Z*-average diameters were calculated as described previously (Herringson and Altin, 2009).

2.7. Production of liposomes, doxorubicin loading and peptide engraftment

Liposomes were prepared with one of the two lipid compositions: DSPC/cholesterol/PE-PEG_{2000}/NTA_3-DTDA (55:39:5:1 molar ratio) or DSPC/cholesterol/PE-PEG_{750}/NTA_3-DTDA (50:39:10:1 molar ratio). For fluorescence studies Texas Red-DHPE also was included at 1 mol%. Lipids were dried under a stream of nitrogen gas, and liposomes produced by suspending the lipids in PBS containing the appropriate amount of Ni-NTA_3-DTDA and sonicating for 45 s (two bursts) at maximum amplitude using a probe sonicator. For radiolabelled liposomes, trace amounts of [4-^{14}C]Cholesterol was added to the lipid mixture and incorporated into liposomes using a bath sonicator (5 min). For loading of doxorubicin, liposomes were prepared in 250 mM ammonium sulphate (pH 5.5) and then subjected to buffer-exchange by loading onto a NICK column containing Sephadex G-50 equilibrated in PBS (pH 8) and eluting in PBS. Doxorubicin was mixed with the liposomes (100 μg drug per μmol lipid) and the mixture incubated at 65 °C for 1.5 h. Doxorubicin–liposome samples were then purified through size-exclusion chromatography (NICK column). All liposomes were engrafted with targeting peptides as described for 3NTA-Caelyx.

2.8. Biodistribution studies

C57BL/6 mice were inoculated subcutaneously (into the back of mice) with 1×10^6 B16-F1 cells in a volume of 50 μL of PBS. Biodistribution studies were performed when the tumor diameter reached 5–10 mm (7–12 days after tumor inoculation). The mice were injected i.v. with peptide-engrafted ^{14}C-cholesterol liposomes (0.4 μmol total lipid, 0.8 μCi total radioactivity). At 24 h post-injection, mice were killed by CO_2 asphyxiation. Tissues were harvested, weighed and solubilised in 1 mL of a solution containing 0.5 M sodium hydroxide, 150 mM sodium chloride and 2% SDS. Solubilization was assisted by heating at 60 °C for 24 h. The solubilized tissue (0.3 mL) was added to 3 mL Emulsifier-Safe scintillant (Perkin-Elmer), and radioactivity (dpm) measured using a LS6500 Multipurpose Scintillation Counter (Beckman Coulter, USA). The results were expressed as the percentage of the injected dose per gram of tissue (%ID/g).

2.9. Intratumoral distribution of fluorescent liposomes

B16-F1 tumor-bearing C57BL/6 mice (inoculated as in biodistribution studies) were injected i.v. with 200 μL of peptide-engrafted Texas Red liposomes (2 mM lipid). To allow the visualization of the functional vasculature, mice were injected with 20 μL of Hoescht 33342 (10 mM) 4 h after liposome injection. After 1–2 min the mice were euthanized and the tumor tissue was harvested, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA) and snap frozen in a slurry of dry ice and ethanol. Using a cryostat (Bright Instruments, Huntingdon, UK) set to −25 °C, tissue sections of 10 μm were obtained and mounted on glass slides. Sections were viewed on Leica DMIRE2 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using 590 nm (Texas Red) or 460 nm (Hoescht 33342) filters.

2.10. Tumor inoculation and tumor growth in mice

C57BL/6 mice were inoculated subcutaneously with 5×10^5 B16-F1 cells in the back of the mice. 3NTA-Caelyx or PE-PEG_{750} liposomes containing 1 mol% NTA_3-DTDA and loaded with doxorubicin (approx. 100 μg doxorubicin per μmol lipid) were engrafted with targeting peptides and injected i.v. (via the tail vein) 10 days after tumor inoculation when the majority of the mice had visible tumors of approx. 3–4 mm diameter. The dose of drug injected as doxorubicin liposomes was 2 mg/kg. Mice were injected every 4 days for a total of 3 injections. Tumor size was measured every 2–3 days with callipers; and any mouse for which the diameter of the tumor reached 15 mm was euthanized. Tumor volume was calculated by using the formula: volume = 0.5(length × width^2) (ElBayoumi and Torchilin, 2009).

2.11. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Student's *t*-test was used for statistical analysis, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of targeting doxorubicin liposomes with different peptides

To target doxorubicin-loaded liposomes containing NTA_3-DTDA to the tumor vasculature, targeting peptides were produced to contain a His-tag, a spacer sequence and a targeting motif with purported affinity for either VEGFR-1 (p39-Flt-1) or neuropilin-1 (p24-NRP-1). Similarly, the targeting peptide p47-LyP-1 was produced to contain the targeting motif LyP-1, previously shown to bind the tumor lymphatic marker p32. The peptides were engrafted onto Caelyx that had been incorporated with NTA_3-DTDA, a procedure we recently showed to promote binding of the Caelyx to cells that express receptors for liposome-anchored targeting moieties (Herringson and Altin, 2010). The engrafted Caelyx was injected i.v. (3 separate injections of 2 mg/kg doxorubicin) into C57BL/6 mice bearing subcutaneous B16-F1 tumors. An analysis of the effects of the treatments, however, indicated that the effect of native Caelyx on tumors was indistinguishable from that of Caelyx engrafted with peptide p49 (Control); and that Caelyx engrafted with p39-Flt-1 or p47-LyP-1 had little if any effect. Caelyx engrafted with p24-NRP-1, on the other hand, induced a slight (but not statistically significant) inhibition of tumor growth by the end of the experiment at Day 25 after tumor inoculation (not shown).

3.2. Some peptides can induce aggregation and leakage of liposomal doxorubicin

During the preparation of the targeted 3NTA-Caelyx formulations for the above experiment we observed that in contrast to the other peptides tested, p39-Flt-1 affected the appearance of the liposome suspension. The addition of p39-Flt-1 to 3NTA-Caelyx resulted in the formation of large aggregates of peptide and liposomes (Fig. 1A). Aggregation was noticeable 5 min after the addition of peptide to the 3NTA-Caelyx, and if left undisturbed the aggregates grew in size, separating from the suspension. The aggregates could be dissociated by vigorous mixing (e.g. before injection into mice); but even immediately after mixing, particle

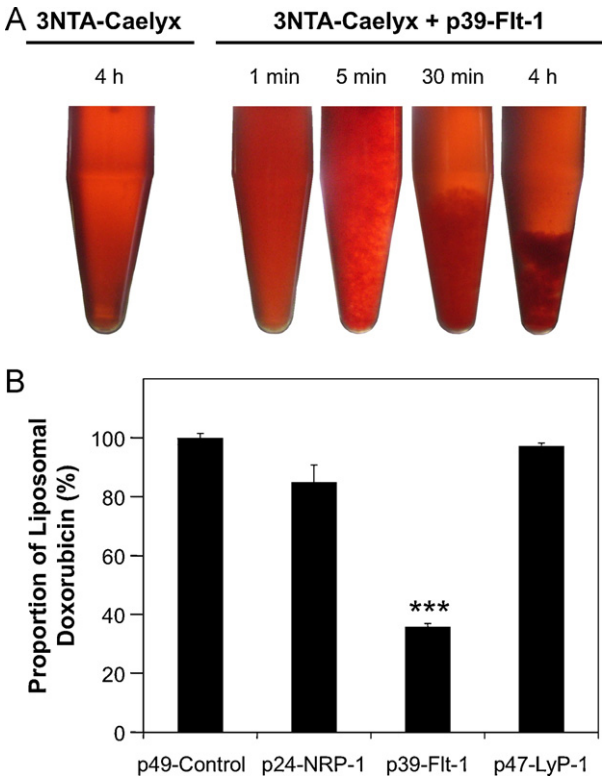

Fig. 1. p39-Flt-1 induces liposomal aggregation and drug leakage. In (A) p39-Flt-1 was added to a suspension of 3NTA-Caelyx and photographed after different periods of time (as indicated). A condition where 3NTA-Caelyx was incubated for 4 h also is included for comparison. In (B) different targeting peptides were added to 3NTA-Caelyx and incubated for 30 min at room temperature. The engrafted liposomes were then subjected to size-exclusion chromatography to determine the proportion of liposome-incorporated doxorubicin. Each bar represents the mean from three experiments ± SEM. $^{***}p < 0.001$ compared to condition p49-Control.

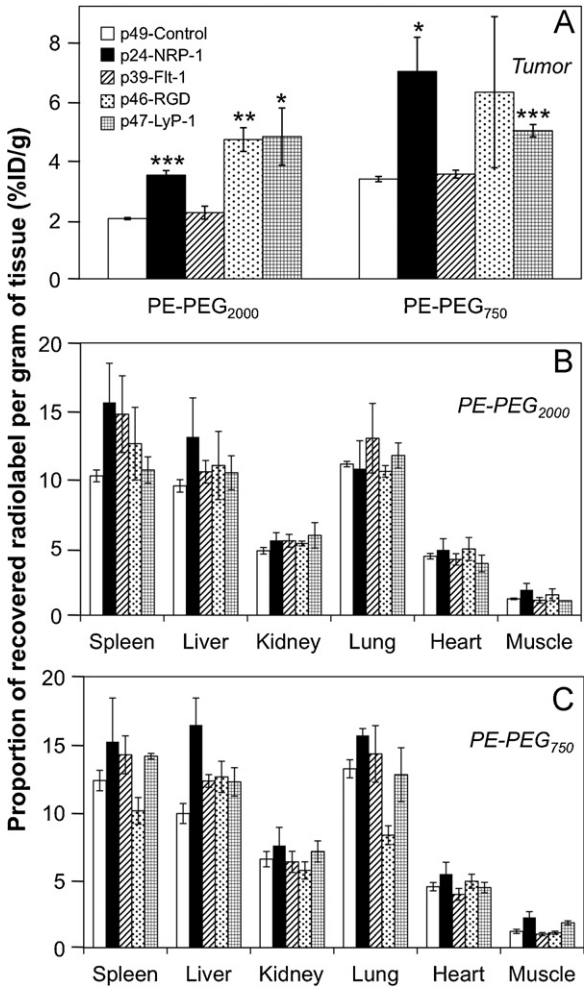

Fig. 2. Tumor accumulation and tissue biodistribution of peptide-engrafted liposomes. Liposomes were prepared with NTA_3-DTDA-containing lipid mixtures with either PE-PEG_{2000} or PE-PEG_{750} (as indicated), and incorporated with the radiolabel [4-^{14}C]-cholesterol as tracer. The liposomes were then engrafted with the targeting peptide indicated, and injected i.v. into different groups of C57BL/6 mice bearing subcutaneous B16-F1 tumors. After 24 h, tumors and tissues (as indicated) were harvested from the mice and the amount of radioactivity associated with each tissue was measured, and the result was expressed as a proportion of the injected radiolabel per gram of tissue. Results for the different conditions are shown in the bar graphs: the results in (A) represent the level of radioactivity in the tumor; whereas the results in (B) and (C) represents the radioactivity in the tissues indicated when using liposomes containing either PE-PEG_{2000} or PE-PEG_{750}, respectively. Each result represents the mean ± SEM from three mice per condition. For panel (A) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ represents significance relative to the respective control (p49-Control) for the indicated experiment.

size measurements with Zetasizer indicated that the diameter of p39-Flt-1-Caelyx was 356 ± 53 nm; compared to 92 ± 3 nm for Caelyx (with or without NTA_3-DTDA modification). Interestingly, the mean particle size for p49-control, p24-NRP-1- and p47-LyP-1-liposomes were all within 125 ± 4 nm. By subjecting the Caelyx to size-exclusion chromatography after incubation at room temperature for 30 min we found that the addition of peptide was accompanied by a release of doxorubicin from the liposomes (Fig. 1B). The results show that while the incorporation of NTA_3-DTDA into Caelyx and the engraftment of p49-Control, p24-NRP-1 or p47-LyP-1 did not cause any significant leakage of doxorubicin, the addition of p39-Flt-1 to the 3NTA-Caelyx led to a substantial release (~65%) of the encapsulated doxorubicin under otherwise identical conditions. p39-Flt-1 induced similar aggregation effects when used with PE-PEG_{750} liposomes (not shown); formulations with p39-Flt-1 were therefore not used in any subsequent experiment.

3.3. Biodistribution of peptide-engrafted liposomes after in vivo administration

Since neither p24-NRP-1 nor p47-LyP-1 induced aggregation or significant release of doxorubicin from the 3NTA-Caelyx, we sought to determine the reason for the somewhat weak ability of these peptides to enhance the anti-tumor efficacy of 3NTA-Caelyx. Biodistribution experiments were carried out to assess the level of liposome accumulation in tumors using liposomes engrafted with the different peptides. Liposomes composed of a lipid formulation comparable to that of 3NTA-Caelyx (DSPC/Chol/PE-PEG_{2000}/NTA_3-DTDA, 55:39:5:1 molar ratio) were produced to contain a trace amount of ^{14}C-cholesterol. The liposomes were engrafted with p49-Control, p24-NRP-1, p39-Flt-1 or p47-LyP-1 and then injected i.v. into B16-F1 tumor-bearing mice. Liposomes engrafted with p46-RGD also were included in these studies for comparison. Experiments in which tissues were assessed at three different times points, namely at 4 h, 24 h and 48 h (not shown) after injection indicated that the highest level of radiolabel in the tumor was recoverable at 24 h post-injection. The proportion of ^{14}C-liposomes (per gram of tissue, %ID/g) measured in the tumors 24 h post-injection is shown in Fig. 2A. It can be seen that the proportion of liposomes detected in the tumors of mice injected with p39-Flt-1 liposomes (2.2 ± 0.2%ID/g) did not differ greatly from tumors in mice injected with p49-Control liposomes (2.0 ± 0.03%ID/g). However, tumors from mice injected with liposomes engrafted with p24-NRP-1, p46-RGD or p47-LyP-1, all showed significant increase

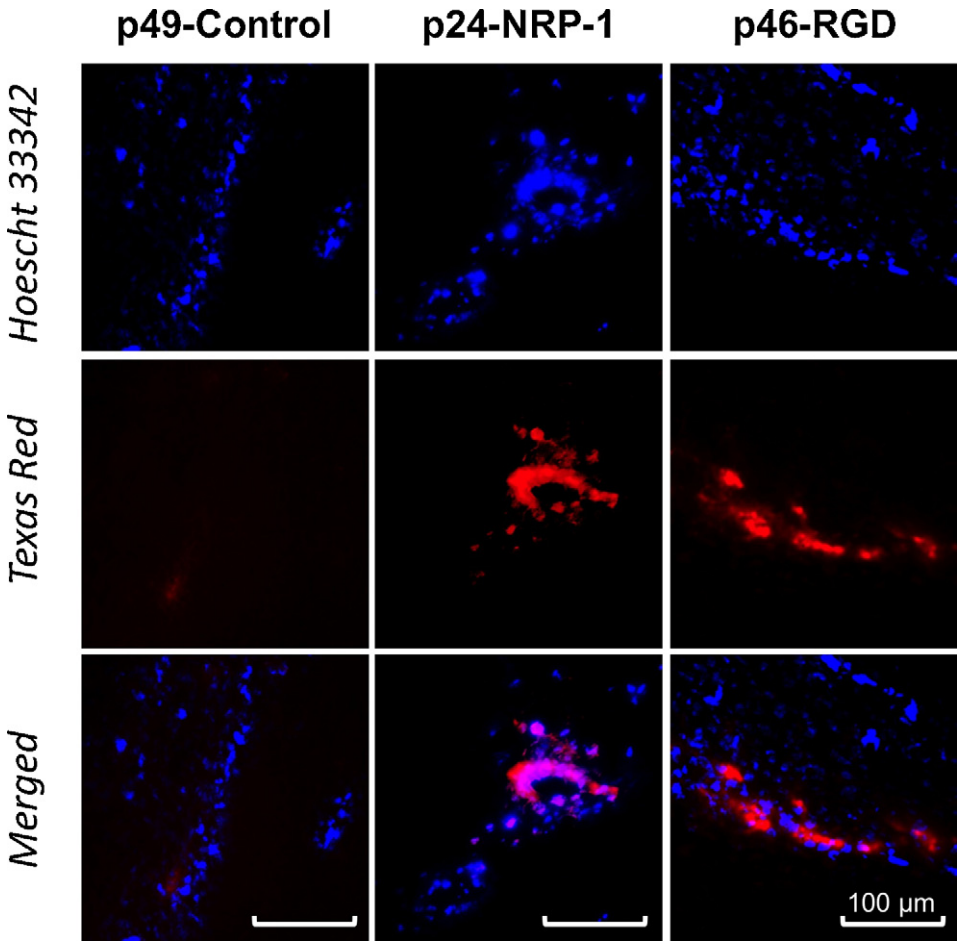

Fig. 3. Intratumoral distribution of peptide-engrafted liposomes. Liposomes with the lipid composition DSPC/cholesterol/PE-PEG_{750}/NTA_3-DTDA/Texas Red-DHPE (mol ratio 49:39:10:1:1) were engrafted with targeting peptides and then injected i.v. into different groups of C57BL/6 mice bearing subcutaneous B16-F1 tumors. After 4 h, mice were injected i.v. with Hoescht 33342 and 1–2 min later the tumor tissue from each mouse was harvested, snap frozen and sectioned. The images shown were captured via fluorescence microscopy and are representative of the fluorescence distribution seen across several tumor sections in two or more independent experiments. Scale bar = 100 μm.

in liposome accumulation: being 3.5 ± 0.2%ID/g, 4.7 ± 0.4%ID/g, and 4.8 ± 1.0%ID/g, respectively (Fig. 2A).

Lipids conjugated to the hydrophilic polymer (PEG) are a critical lipid component of Caelyx (which contains 5 mol% PE-PEG_{2000}), since these lipids promote liposome stability and prolong half-life in the blood circulation (Immordino et al., 2006). However, due to steric effects, PEG-conjugated lipids also have the potential to interfere with interactions between peptides attached directly to the liposome surface and receptors on the surface of the cells being targeted (Torchilin et al., 2001). To determine whether the steric effects of the PEG_{2000} were interfering with the targeting of peptide-engrafted liposomes, we carried out biodistribution experiments with liposomes containing PE-PEG_{750} instead of PE-PEG_{2000}. We used 10 mol% (instead of 5 mol%) PEG_{750} to compensate for the higher non-specific liposome binding due to the lower shielding effect of the shorter PEG_{750} chains. The results in Fig. 2A show that liposomes containing PE-PEG_{750} accumulated in tumors to a significantly greater extent: ~2-fold more for liposomes engrafted with p24-NRP-1, and ~1.5-fold more for liposomes engrafted with p49-Control and p47-LyP-1, compared to liposomes containing PE-PEG_{2000} and engrafted with the same peptides. This effect was not observed for liposomes engrafted with p47-LyP-1. Interestingly, in one mouse injected with p46-RGD PEG_{750} liposomes, 11.1%ID/g was detected in the tumor; but the average that accumulated in tumors did not differ significantly between the two liposome formulations. When compared to p49-Control PEG_{750} liposomes (3.4 ± 0.1%ID/g), both p24-NRP-1 and p47-LyP-1 liposomes accumulated to a significantly greater extent in the tumor: being 7.0 ± 1.2%ID/g and 5.0 ± 0.2%ID/g, respectively (Fig. 2).

The proportion of the injected dose recovered in other tissues such as the spleen, liver, lungs, heart, kidney and muscle is shown in Fig. 2B and C. From the results it can be seen that regardless of the targeting peptide or liposome composition, the greatest accumulation of radiolabelled liposomes occurred in the spleen (10–16%ID/g), liver (10–16%ID/g) and lungs (8–16%ID/g). Although the liposomal accumulation in each tissue type did vary somewhat depending on the targeting peptide used, typically there was no significant difference in accumulation when compared with p49-Control liposomes. Interestingly, a comparison of the distribution data obtained using liposomes containing the two different PEG-lipids (Fig. 2B and C) indicated that PEG_{750} liposomes accumulated to a significantly higher level in the lungs compared to PEG_{2000} liposomes, when the liposomes were engrafted either with p49-Control (PEG_{2000}: 11.1 ± 0.2%ID/g; PEG_{750}: 13.2 ± 0.6%ID/g) or with p24-NRP-1 (PEG_{2000}: 9.7 ± 2.0%ID/g; PEG_{750}: 15.6 ± 0.5%ID/g) peptide. Importantly, a comparison of the results in Fig. 2B and C revealed that, apart from the p49-Control PEG_{2000} condition, all peptide-engrafted liposomes accumulated >2-fold more in the tumor, than in muscle. The largest tumor:muscle ratio (4.4) occurred for p47-LyP-1 PEG_{2000} liposomes (Fig. 2B).

3.4. Intratumoral distribution of peptide-engrafted liposomes

Since both p24-NRP-1 and p47-LyP-1 liposomes accumulated into tumors to a greater extent compared to p49-Control liposomes, experiments were carried out to determine the localisation of the liposomes within the tumor. We prepared PEG_{750} liposomes containing NTA_3-DTDA as well as a fluorescent lipid marker (Texas Red-DHPE), engrafted these liposomes with targeting peptides, and then injected them into mice bearing subcutaneous B16-F1 tumors. After 4 h, the tumor tissue was harvested and sections were prepared for analysis by fluorescence microscopy. While little or no liposomal (red) fluorescence was seen in tumors from mice injected with p49-Control liposomes, areas of strong fluorescence could be seen in the tumors of mice injected with p24-NRP-1 liposomes (Fig. 3). Importantly, this liposome-associated fluorescence overlapped with areas of the tumor that were directly accessible to the blood flow, as reflected by the blue fluorescence due to the injected stain Hoescht 33342. For tumors from mice injected with p24-NRP-1 liposomes, there were no detectable areas where strong liposomal fluorescence occurred in the absence of Hoescht 33342 fluorescence. Similar results also were obtained when liposomes engrafted with p46-RGD were injected into mice (Fig. 3). The results in Fig. 4 show the intratumoral distribution of p47-LyP-1 liposomes after injection into B16-F1 tumor-bearing mice. Although there were areas where liposomal fluorescence and Hoescht 33342 fluorescence could clearly be seen to overlap, distinct regions (indicated with arrows) where liposomes accumulated away from the functional vasculature of the tumor also could be observed (Fig. 4).

3.5. Anti-tumor effect of peptide-engrafted PE-PEG_{750} doxorubicin-loaded liposomes

Since biodistribution studies indicated that peptide-engrafted liposomes containing PE-PEG_{750} accumulate in tumors to an equal or sometimes greater extent compared to liposomes containing PE-PEG_{2000}, experiments were carried out to determine whether peptide-engrafted PEG_{750} liposomes loaded with doxorubicin could induce a targeted anti-tumor effect. Doxorubicin-loaded PEG_{750} liposomes were engrafted with p49-Control, p24-NRP-1 or p47-LyP-1 before i.v. injection into C57BL/6 mice bearing subcutaneous B16-F1 tumors (3 separate injections of 2 mg/kg doxorubicin on Day 10, 14 and 18 after tumor inoculation). Importantly, this dose schedule of treatment was the same as that followed for experiments with 3NTA-Caelyx (Section 3.1). The effect of doxorubicin alone was not explored in this system since free doxorubicin is much more cardiotoxic than liposomal doxorubicin; and liposomal, but not free doxorubicin, has the potential to be efficiently targeted to cells by receptor-mediated targeting of loaded liposomes. As shown in Fig. 5, when compared to liposomes engrafted with p49-Control, treatment with p24-NRP-1 liposomes caused a significant inhibition in the rate of tumor growth, so that by Day 23 the mean tumor size of the p24-NRP-1 group ($918 \pm 58\ mm^3$) was only 68% of the size of that of the p49-Control group ($1350 \pm 138\ mm^3$). The administration of p47-LyP-1 liposomes containing doxorubicin, however, did not significantly affect the rate of tumor growth when compared to mice treated with non-targeted liposomes (Fig. 5).

4. Discussion

In this work three different targeting peptides containing sequences purported to bind cell markers expressed in the tumor vasculature (p24-NRP-1 and p39-Flt-1) (An et al., 2004; Starzec et al., 2006) and tumor lymphatics (p47-LyP-1) (Laakkonen et al., 2002), were examined for their ability to target NTA_3-DTDA-containing liposomes to subcutaneous B16-F1 tumors. Importantly,

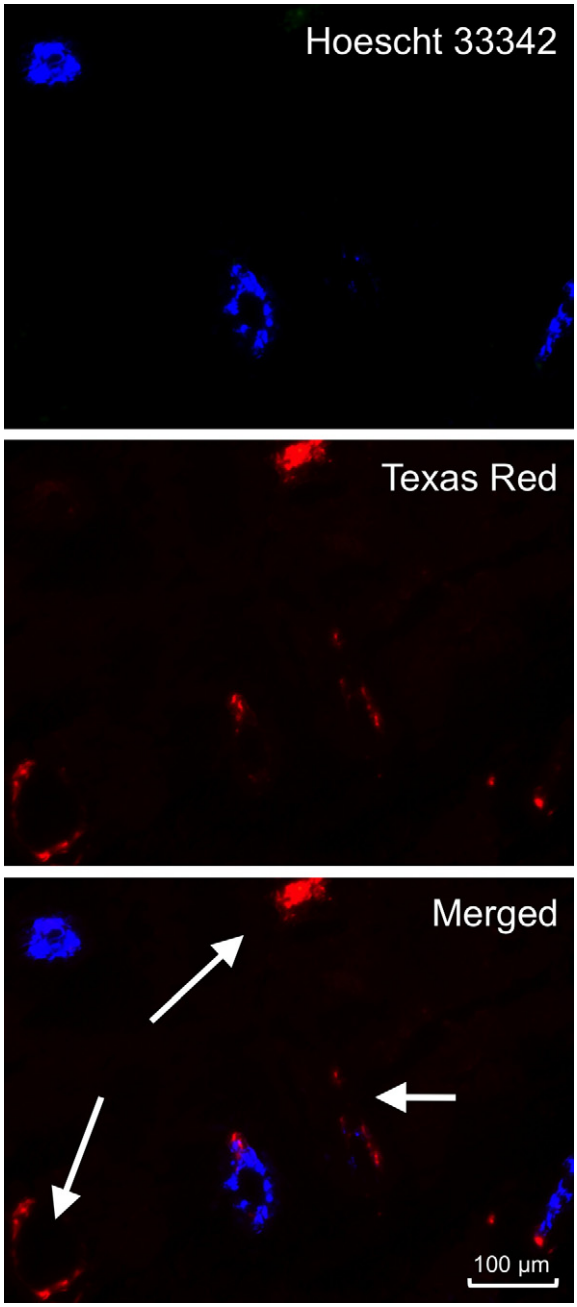

Fig. 4. Intratumoral distribution of p47-LyP-1-engrafted liposomes. Liposomes with the lipid composition DSPC/cholesterol/PE-PEG_{750}/NTA_3-DTDA/Texas Red-DHPE (mol ratio 49:39:10:1:1) were engrafted with p47-LyP-1 and then injected i.v. into C57BL/6 mice bearing subcutaneous B16-F1 tumors. Images of fluorescent areas of the tumor were taken as described in the legend to Fig. 5. Scale bar = 100 μm.

using this approach a significant anti-tumor effect was seen after the administration of doxorubicin-loaded PEG_{750} liposomes engrafted with p24-NRP-1.

Our initial studies showed that the peptide p39-Flt-1, despite containing a targeting motif that was previously demonstrated to bind VEGFR-1 (An et al., 2004; Herringson et al., 2009), did not promote any significant accumulation of radiolabelled liposomes in tumors (Fig. 2). Consistent with this observation, minimal effect was seen after i.v. administration of p39-Flt-1-engrafted 3NTA-Caelyx (not shown). While the reason for the inability of p39-Flt-1 liposomes to target tumors was not explored, it was noted that the addition of p39-Flt-1 to a suspension of 3NTA-Caelyx increased particle size, promoted aggregation, and induced substantial leakage of

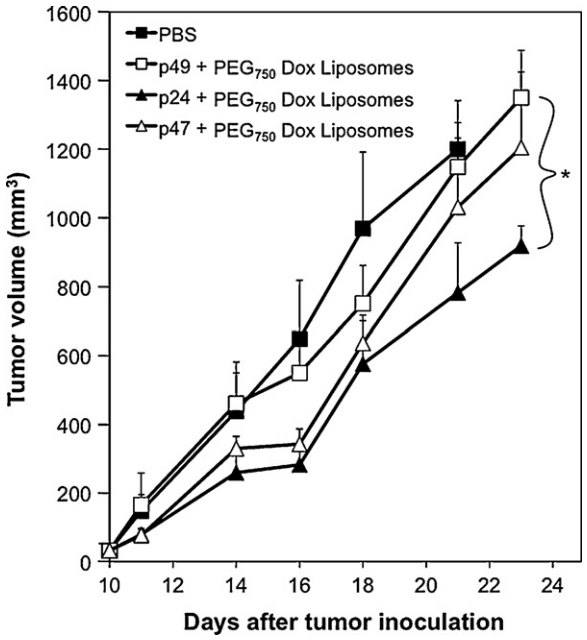

Fig. 5. Effect of doxorubicin-loaded liposomes containing PE-PEG_{750} on B16-F1 tumor growth. Doxorubicin-loaded liposomes were prepared with the lipid composition DSPC/cholesterol/PE-PEG_{750}/NTA_3-DTDA (mol ratio 50:39:10:1). Liposomes were engrafted with p49-Control, p24-NGR-1 or p47-LyP-1. C57BL/6 mice ($n = 6$) bearing subcutaneous B16-F1 tumors were injected i.v. with PBS or peptide-engrafted doxorubicin-loaded liposomes (2 mg/kg doxorubicin) on Day 10, 14 and 18 after tumor inoculation. Tumor size was measured every 2–3 days and the mean tumor size at each time point is shown. Results for condition with un-engrafted doxorubicin liposomes (without NTA_3-DTDA) were similar to those for liposomes engrafted with p49-Control and were omitted for clarity. Error bars indicate the SEM. $^*p < 0.05$.

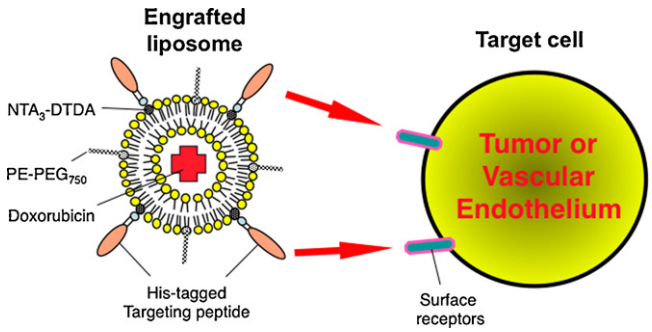

Fig. 6. Peptide-engrafted liposomes target drug to tumors. The simplified diagram shows how the chelator lipid NTA_3-DTDA [3(nitrilotriacetic-acid)-ditetradecylamine] incorporated into liposomes [composed primarily of a carrier lipid such as phosphatidylcholine, cholesterol and stealth lipid PE-PEG_{750} (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-polyethylene glycol-750] can be used to anchor His-tagged peptide moieties for targeting liposome-encapsulated drugs such as doxorubicin to specific receptors on either tumor cells or on vascular endothelial cells within the tumor, to enhance the cytotoxic effects of the drug.

free doxorubicin from the Caelyx (Fig. 1). The mechanism for these effects is unclear, but most likely involves the highly hydrophobic nature of the p39-Flt-1 targeting motif (which contains three tryptophans, two leucines and a methionine). It is likely that p39-Flt-1 aggregates/disrupts liposomes by the His-tag binding to NTA_3-DTDA on one liposome, while the hydrophobic targeting motif simultaneously interacts with or perturbs the bilayer of adjacent liposomes.

In contrast to the effects seen with p39-Flt-1, no significant aggregation or drug leakage from the liposomes was observed when p24-NRP-1 was added to 3NTA-Caelyx, suggesting that under these conditions the somewhat lower hydrophobicity of the targeting moiety of p24-NRP-1 (which contains five hydrophobic residues) does not cause significant liposome aggregation/disruption. Interestingly, the conjugation of antibodies onto liposomes also has been reported to induce aggregation and subsequent rupture of liposomes (Bredehorst et al., 1986). Such antibody-mediated liposome aggregation, could readily be overcome, however, by the inclusion of PEG-conjugated lipids (Harasym et al., 1995).

PEG-conjugated lipids are known to create a steric barrier around liposomes, which reduces their ability to aggregate and interact with plasma components, and extends the lifetime of the liposomes in the blood circulation (Torchilin, 2005). However, the long PEG_{2000} chains may also interfere with the interaction of liposome-anchored moieties to receptors when using PEGylated lipids (Klibanov et al., 1991; Park et al., 1995; Hansen et al., 1995). This effect is highlighted in the present work using NTA_3-DTDA-liposomes, where the presence of PE-PEG_{2000} in the Caelyx clearly limited its ability to be targeted by p24-NRP-1 to the tumor vasculature. Although radiolabelled PEG_{2000} liposomes engrafted with p24-NRP-1 did exhibit increased tumor accumulation (3.5%ID/g compared to 2%ID/g for p49-Control liposomes), it appears that the amount of p24-NRP-1-engrafted Caelyx that reached the tumor under these conditions elicited only a small if any inhibition on tumor growth. Importantly, however, the proportion of p24-NRP-1 liposomes that accumulated in tumors increased by 2-fold (compared to the non-targeted control) when liposomes were prepared to contain PE-PEG_{750} instead of PE-PEG_{2000} (Fig. 2). Consequently, compared to mice treated with non-targeted liposomes, there was a clear inhibition in the rate of tumor growth in mice treated with doxorubicin-loaded PEG_{750} liposomes engrafted with p24-NRP-1 (Fig. 5). The anti-tumor effect seen after three administrations of p24-NRP-1-engrafted PEG_{750}-doxorubicin liposomes was not dramatic, but nonetheless significant—as indicated in Fig. 5. A more dramatic effect against tumors could well have been obtained, however, if additional administrations and/or if treatment had been continued for a longer period of time; this was not explored in this study due to animal ethics considerations—issues likely to have less relevance to patients with intractable cancer.

Interestingly, the proportion of p47-LyP-1 liposomes that accumulated in the tumor (~5%ID/g) was not affected by whether the liposomes contained PE-PEG_{2000} or PE-PEG_{750}. It is unclear therefore whether both types of sterically stabilizing lipid interfered with the interaction of p47-LyP-1 with p32. To reduce the possibility of the PEG chains interfering with peptide–receptor interactions, targeting peptides are often attached to the distal end of the PEG chains of PEG-lipids, rather than directly onto the liposomal surface (Negishi et al., 2010; Zalipsky et al., 1995). Our approach using the chelator lipid NTA_3-DTDA, nonetheless, is different. We envisage that the combination of a molecular spacer between the membrane anchor and 3NTA headgroup of this lipid, and a spacer/linker region between the His-tag and targeting moiety of the peptide (Altin et al., 2006), would enable sufficient projection of the engrafted peptide to minimise steric effects due to the PEG_{750} on the liposomes. Interestingly, a recent study where the LyP-1 sequence was conjugated to liposomal doxorubicin via maleimide-derivatized PE-PEG_{2000}, also reports no substantial benefit or anti-tumor efficacy compared to non-targeted liposomes (Park et al., 2010). It would appear, therefore, that factors other than steric hindrance, such as a lower level of expression of Lyp-1 receptors (p32) on B16-F1 tumors, are likely to play a role in negating the therapeutic potential of p47-LyP-1-Caelyx in this system.

The difference between the anti-tumor effect observed herein for p47-LyP-1-Caelyx and that for p24-NRP-1 (Fig. 5) could reflect differences between the drug-delivery strategies employed; that is, the targeting of tumor cells directly, versus targeting of the tumor vasculature. It can be expected that cytotoxic drugs targeted specifically to tumor cells will kill only the cells that they come into direct contact with. In contrast, since tumor growth is dependent on a functional blood supply, the death of one tumor endothelial cell could well result in the killing of up to 100 tumor cells (Binetruy-Tournaire et al., 2000). For similar therapeutic effect, therefore, liposomes targeted directly to tumor cells with p47-Lyp-1 would require a greater tumor accumulation compared to vasculature-targeted liposomes. However, the proportion of administered p47-LyP-1 liposomes that accumulated in tumors was similar to that of the vasculature-targeted p49-RGD liposomes (approx. 5%ID/g). This observation is consistent with these considerations and suggests that the accumulation that occurred with p47-LyP-1 was insufficient to elicit a detectable tumor response under these conditions. Interestingly, a recent report has shown that the application of tumor-specific hyperthermia can increase the tumor accumulation of LyP-1-targeted liposomal doxorubicin, leading to complete tumor regression (Park et al., 2010). Clearly, the use of hyperthermia or other approaches that increase the accumulation of liposomes in tumors could also yield a similar outcome when combined with treatment with 3NTA-Caelyx engrafted with p47-LyP-1.

The present work also analysed the intratumoral distribution of peptide-engrafted liposomes after in vivo administration. It is reported that i.v. injection of the dye Hoescht 33342 results in a rapid uptake by cells without diffusion across cell layers; this provides a useful tool for identifying functional vasculature (Smith et al., 1988). Consistent with the purported ability of the targeting motif of p24-NRP-1 to target delivery to the tumor vasculature, an examination of the fluorescence of tumor sections revealed that p24-NRP-1 liposomes localised primarily in areas that overlapped with areas of strong Hoescht 33342 fluorescence (Fig. 3). Interestingly, our results also show that p47-LyP-1 liposomes accumulate in cells surrounding the tumor vasculature as well as in extravascular regions of the tumor (Fig. 4). These findings contrast those of others using LyP-1 targeted nanoparticles including micelles, baculovirus particles and inorganic azido-nanoparticles, where no accumulation was demonstrated in tumor blood vessels (Karmali et al., 2009; Makela et al., 2008; von Maltzahn et al., 2008). While these differences may be due to the nature of the particles being used, they also may reflect tumor-specific differences between the murine B16-F1 melanoma used herein, versus the human MDA-MB-435 tumors used by others (Karmali et al., 2009; Makela et al., 2008; von Maltzahn et al., 2008).

In relation to clinical use, we note that Caelyx, as the marketed liposomal formulation of doxorubicin, has very good stability when stored refrigerated; and we therefore expect that a form of Caelyx produced to contain both PE-PEG_{750} (instead of PE-PEG_{2000}) and NTA_3-DTDA (to enable targeting with peptides), would be similarly stable under those conditions. In view of the potential for certain peptides to cause drug leakage from liposomes (Fig. 1), however, we would advocate that targeting peptides first be screened or selected for compatibility with tumor targeting, and that the targeting peptide then be mixed with the NTA_3-DTDA-containing liposomal doxorubicin for some 30 min to allow engraftment before administering to patients. Similar to Caelyx, such peptide-targeted liposomal doxorubicin should be administered by the i.v. route. We envisage that new formulations of targeted liposomal doxorubicin similar to those described herein (e.g. p24 + $PEG7_{50}$Dox-Liposomes) would be useful in all applications for which liposomal doxorubicin is indicated; but expect that the targeted formulations would be significantly more efficacious.

5. Conclusions

This work has shown that liposomes incorporated with the chelator lipid NTA_3-DTDA provide a convenient way to anchor peptide targeting moieties to target liposomes/liposomal drugs to tumors (Fig. 6). Thus, NTA_3-DTDA can be incorporated into liposomes loaded with the anticancer drug doxorubicin, allowing convenient engraftment of histidine-tagged targeting peptides. Three peptides p39-Flt-1, p24-NRP-1 and p47-LyP-1 purported to contain moieties that can target tumors, were examined for their ability to target liposomal doxorubicin. Our results show that the use of some peptides (e.g. p39-Flt-1) can be problematic by promoting liposome aggregation and/or leakage of the encapsulated liposomal drug. Importantly, however, by careful selection of the lipid formulation in respect to the PEG-lipid used, peptides can readily be screened to identify those suitable for therapeutic targeting of liposomes to tumors. This was demonstrated by our finding of a significant anti-tumor response in mice bearing subcutaneous B16-F1 tumors treated with p24-NRP-1-Caelyx. We envisage that this same approach has potential for targeting and enhancing the efficacy of liposomal drugs used to treat cancer in humans.

Acknowledgements

The authors are grateful to Abdus Faham for his expert technical assistance with some of the experiments. This work was supported by Project Grant (No. 316 949) to J.G.A. from the NHMRC of Australia. The authors wish to thank Lipotek Pty Ltd for providing the NTA_3-DTDA lipid for research purposes. J.G.A. declares a commercial interest in Lipotek Pty Ltd.

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