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ORIGINAL ARTICLE

Targeting liposomes loaded with melphalan prodrug to tumour vasculature via the Sialyl Lewis X selectin ligand

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Abstract

Earlier we showed that liposome formulation of DL-melphalan lipophilic prodrug bearing tetrasaccharide Sialyl Lewis X (SiaLe^X) caused prolonged therapeutic effect on mammary cancer in mice. Here, we compare antivascular effect of SiaLe^X-liposomes loaded with diglyceride ester of melphalan (Mlph) against SiaLe^X-free formulation in Lewis lung carcinoma model.
Methods: Liposomes of egg phosphatidylcholine/yeast phosphatidylinositol/1,2-dioleoyl glycerol (DOG) conjugate of Mlph/$\pm$SiaLe^X-PEG$_{8-15}$-DOG, 8:1:1:0.2 by mol, were prepared by standard extrusion. After two intravenous injections with Mlph or liposomes under either standard or delayed treatment protocols, vascular-disrupting effects of the preparations were evaluated basing on tumour section histomorphology, lectin perfusion assay and immunohistochemistry (anti-CD31 staining) data. Also, untreated mice were administered with fluorescently-labelled liposomes to assess their distribution in tumour sections with confocal laser scanning microscopy.
Results: Two injections of SiaLe^X-liposomes reproducibly caused severe injuries of tumour vessels. SiaLe^X-liposomes co-localized with CD31 marker on vascular endothelium while the non-targeted formulation extravasated into tumour.
Discussion: Cytotoxic SiaLe^X-liposomes exhibit superior vascular-disrupting properties compared to non-targeted liposomes, yet the effect starts to transform into gain in tumour growth inhibition only under delayed treatment regimen.
Conclusion: SiaLe^X-ligand provides targeting of cytotoxic liposomes to tumour endothelium and subsequent antivascular effect.

Keywords

Antivascular effect, drug delivery, Lewis lung carcinoma, lipophilic prodrugs, melphalan, nanomedicine, tumour endothelium

History

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Introduction

Delivery of nanosized liposomes to tumour cells via specific receptors – i.e. active targeting – has been proven feasible for treatment of some malignant diseases [e.g. 1]. Although, targeting continues to be a challenge in each particular case: tumour cells are well protected from the reach of the therapeutic units circulating in bloodstream by the vascular and interstitial barriers; they differentiate into various clones of malignant cells, including those resistant to the drug, creating spatial and temporal heterogeneity in tumour tissue and thus precluding the success of therapy specifically targeted at primary tumour cells. Targeting supporting cells of tumour tissue that ensure its survival and growth has evolved to overcome these obstacles (as reviewed in [2]). Liposomal formulations proposed to target endothelial cells include liposomes decorated with RGD peptide to target $\alpha\nu$-integrins [3,4] and liposomes covalently linked with anti-VEGFR2 antibody, to target VEGF receptor-2 [e.g. 5].

Rather recently, selectins have been recognized as another prospective target for delivery to tumour endothelial cells [6,7]. Selectins are carbohydrate binding adhesion proteins expressed on the luminal surface of activated endothelial cells (E- and P-selectins), circulating leukocytes (L-selectin) and activated platelets (P-selectin). Through mediation of leukocyte tethering and rolling and endothelium activation, selectins play the key roles in multiple (patho)physiological processes, including inflammatory responses and metastasis development [6,8–10]. Recently, the crucial role of E- and P-selectins in spontaneous metastasis formation was evidenced *in vivo* in a mouse model of colon cancer [9]. A study by [11], employing E-selectin knock-down mice, provided strong evidence for E-selectin being a potent target for inhibition of angiogenesis and tumour growth at least in melanoma treatment. A common carbohydrate epitope recognized by selectins of all types is the Sialyl Lewis X tetrasaccharide (SiaLe^X, Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ) [12]. This provides basic rationale for targeting drugs to tumours and inflammation foci via the SiaLe^X ligand.

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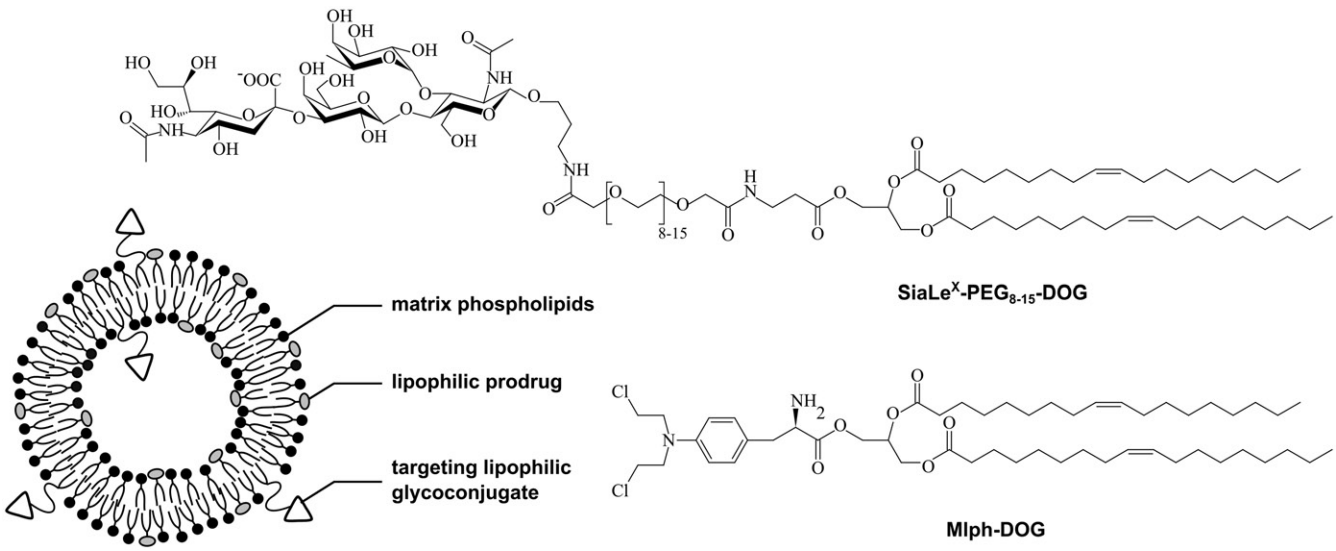

Figure 1. Schematic representation of a liposome loaded with a lipophilic prodrug and a targeting tetrasaccharide conjugate in the bilayer and chemical structures of the lipophilic conjugates of melphalan (Mlph-DOG) and Sialyl Lewis X (SiaLe^X-PEG$_{8–15}$-DOG) used in this work.

Earlier, we showed that liposomes carrying a lipophilic prodrug of sarcolysin (DL-melphalan) and equipped with SiaLe^X targeting ligand caused prolonged therapeutic effect on breast cancer in mice: the targeted liposomes increased survival twice as efficiently as the ligand-free formulation and four times more efficiently than sarcolysin as such [13]. We hypothesize that, in addition to killing malignant cells, the targeted liposomes blocked tumour vascularization, which inhibited tumour growth. The goal of this study was to examine the possible antivascular effect of SiaLe^X-bearing cytotoxic liposomes.

The liposomal formulations under study (Figure 1) are designed to reliably incorporate water soluble chemotherapeutics in the form of lipophilic prodrugs [14]. The lipid bilayer is formed of fluid-phase lipids and contains phosphatidylinositol as an anti-opsonizing component to reduce uptake by the reticuloendothelial system [15,16]. The liposomes were well tolerated by the major cellular components of blood independently of the presence of SiaLe^X epitope [17], confirming no adhesion of selectin ligand-decorated particles to non-activated platelets. In the current study, we compare vascular-disrupting potential and intratumoural localization of SiaLe^X-liposomes loaded with the lipophilic prodrug of melphalan (Figure 1) versus its SiaLe^X-free counterpart in the model of Lewis lung carcinoma.

Methods

Reagents and chemicals

Phosphatidylcholine (PC) from egg yolk and phosphatidylinositol (PI) from *S. cerevisiae* were obtained from Reakhim (Russia). A conjugate of a tetrasaccharide Sialyl Lewis X 3-aminopropyl glycoside and *rac*-1,2-dioleoyl-3-carboxymethylene[poly(8-15)oxyethylene]oxyacetylamidopropionylglycerol (SiaLe^X-PEG$_{8-15}$-DOG) [18], 1,2-dioleoylglyceride ester conjugate of melphalan (Mlph-DOG) [19] and 1-palmitoyl-2-[7-(Me4-BODIPY-8)heptanoyl]-*sn*-glycero-3-phosphocholine (BODIPY-PC) [20] were synthesized as previously reported.

Liposome preparation

Liposomes composed of PC, PI and Mlph-DOG (8:1:1 molar ratio), either equipped with 2 mol. % glycoconjugate SiaLe^X-PEG$_{8–15}$-DOG or not, were prepared as described earlier [17]. Briefly, lipid films, typically containing 42.9 mg PC, 6.0 mg PI, 6.23 mg (6.9 μmol) of Mlph-DOG and, optionally, 3.0 mg SiaLe^X-PEG$_{8–15}$-DOG, were hydrated in 3 mL PBS (1.5 mM KH_2PO_4, 1.1 mM $NaH_2PO_4 \cdot 2H_2O$, 6.3 mM Na_2HPO_4, 2.7 mM KCl and 136.8 mM NaCl, pH 7.2) containing 1 mM EDTA and subjected to 6–10 cycles of freezing/thawing (liquid nitrogen/+40°C). The suspension was then extruded at ambient temperature through two stacked polycarbonate membrane filters of 100 nm (Nucleopore), 10 times through each pair, on a Mini-extruder (Avanti Polar Lipids, Alabaster, AL). Also, SiaLe^X-containing liposome formulation without Mlph-DOG (PC–PI–SiaLe^X-PEG$_{8–15}$-DOG, 8:1:0.2, by mole) was prepared in the same manner.

In dispersions, prodrug concentration was determined upon liposome disruption with at least 20-fold volume of ethanol by spectrophotometry (Mlph-DOG: λ_{max} 258 nm, ε ~19 700 $M^{-1}cm^{-1}$) on an SF-256-UVI two-beam spectrophotometer (LOMO Fotonika, Russia).

Some liposome samples were prepared labelled with fluorescent BODIPY-PC added at the stage of lipid film formation (0.5 mol. %). Formulations were stored at 4°C and used for biological experiments within 3 days.

Liposome physical characteristics

Liposome size upon preparation was controlled in diluted suspensions (50 μg total lipids/mL PBS) by dynamic light scattering using a 90Plus (Brookhaven Instruments Corp., Holtsville, NY) equipment in at least three runs per sample.

Zeta potential values were obtained using a 90Plus (Brookhaven Instruments Corp., Holtsville, NY) PALS mode for 100-nm liposomes prepared in 10 mM KCl solution buffered with 1 mM K^+/Na^+-phosphate buffer, pH 7.2, at concentration of 0.9–1.0 mg lipid/mL. Samples were equilibrated for 1 min in pre-rinsed disposable cuvettes before a

minimum of six runs of 30 cycles per sample was performed at 25°C. Zeta potential values were calculated using Smoluchowski approximation.

Mice

Male BDF1(C57Bl6*DBA\2) mice aged 4–5 weeks weighing 27.3 ± 1.9 g were inoculated subcutaneously with 10^6 Lewis lung carcinoma (LLC) cells suspended in 300 μL 199 medium in the axillary cavity. Single-cell suspension of a tumour excised from a donor animal was prepared according to standard procedures. Briefly, after excision from a donor animal, LLC tumour was minced, passed through stainless steel mesh with decreasing pore size and treated with 0.1% collagenase for 30 min to obtain a single-sell suspension, which was further washed three times with 199 medium. Cell viability and concentration were determined upon trypan blue staining and counting in Goryaev chamber under light microscope.

The animal experiment protocols were approved by the Committee for Ethics of Animal Experimentation and the experiments were conducted in accordance with the Guidelines for Animal Experiments in N.N. Blokhin Russian Cancer Research Center, Russian Academy of Medical Sciences.

Antitumour effect of the preparations (against the primary nodule) was evaluated by the dynamics of tumour growth assessed on day 7 after tumour transplantation and each 3–5 days further on (tumour growth inhibition, %). Tumours were measured with the aid of a metric calliper and tumour volume was calculated as the product of three perpendicular diameters of the tumour.

Tumour growth inhibition was determined according to the formula: $(1 - V_{exp}/V_{ctrl}) \times 100\%$, where V_{exp} is tumour volume in treated animals and V_{ctrl}, in the control. By the end of the experiments, the average body weight over all groups was 28.3 ± 2.5 g. Significance of the data obtained was evaluated by two-tailed unpaired Student's *t*-test.

Early treatment (standard) protocol

On days 3 and 7 after tumour inoculation, mice ($n = 10$ per group) were given intratail injections (0.25 mL) of the following preparations: PBS (control), melphalan (2.3 mM, i.e. 175 μg per mouse, which is ~7 mg/kg), liposomes loaded with Mlph-DOG (2.3 mM; sample L-Mlph-DOG) and SiaLe^X-liposomes loaded with Mlph-DOG (2.3 mM; sample SiaLe^X-L-Mlph-DOG). Samples for histology, perfusion and vessel count studies were collected on days 9 and 15.

Delayed treatment protocol

Mice ($n = 9$ per group) were treated with the same preparations as under the early treatment regimen, but on days 7 and 10 after tumour inoculation. An additional group received control liposomes bearing SiaLe^X-PEG$_{8–15}$-DOG (sample SiaLe^X-L, $n = 9$) and no Mlph-DOG. Samples for perfusion and vessel count studies were collected on day 12.

Histology, intravital perfusion and immunohistochemistry

Mice ($n = 3$ from each group) were sacrificed with ether overdose. Ten minutes before euthanasia, they were injected with 150 μL 0.01 mg/mL biotinylated *Lycopersicon esculentum* lectin (LEL; Vector Laboratories, Burlingame, CA) in the tail vein. Excised tumours were rinsed in ice-cold PBS and fixed in 10% neutral formalin for 24 h. Fixed tissues were embedded in paraffin and sectioned. Five-micrometer sections were stained with either haematoxylin and eosin (H&E) for histomorphology analysis, or avidin–biotin peroxidase complex (Vector Laboratories, Burlingame, CA) followed by diaminobenzidine (Sigma, St Louis, MO) to visualize LEL, or immunostained with rabbit polyclonal antibodies to CD31 (Abcam, UK). Bound primary antibodies were detected with anti-rabbit Labelled Polymer HRP (EnVision HRP; Dako). LEL- and CD31-stained sections were counterstained with hematoxylin and mounted.

Tumour vessel count

Vessels and capillaries in histological sections were identified by CD31 staining and appropriate morphology and counted as described previously [21,22]. Two sections of each tumour, five fields of vision in each, were imaged and quantified using an AxioVision software (Carl Zeiss Imaging Systems). To assess antiangiogenic/antivascular properties of the formulations, inhibition of vessel growth was calculated as percent difference in the number of CD31-positive microvessels in the experimental samples compared with the control samples. LEL-perfused vessels were counted in the same manner to assess the number of functional tumour vessels upon treatment. All counts were performed twice, by two researchers (ES and DK) independently of each other.

Fluorescence microscopy imaging

To study intratumoural localization of liposome formulations, on day 7 after tumour transplantation groups of untreated mice ($n = 3$) were progressively injected (i.v.) with BODIPY-PC-labelled liposomes and Hoechst 33342 (Thermo Scientific) solution (15 mg/kg) with an interval of 20 min. After another 20 min, mice were ether euthanized, tumours were excised, fixed in 4% formalin, soaked progressively in 5–20% glucose and finally embedded in the *Killik* frozen section *medium* (Bio Optic, Milan, Italy) and frozen in isopentane cooled in liquid nitrogen. For confocal laser scanning microscopy (CLSM) analysis with Nikon TE-2000 Eclipse (Japan) microscope, cryosections were treated with primary rabbit polyclonal anti-CD31 antibodies (Abcam PLC) and visualized with secondary Alexa Fluor® 594 goat anti-rabbit IgG (H + L) (Invitrogen, Grand Island, NY). Alexa Fluor® 594 CD31 (λex 590 nm and λem 617 nm), BODIPY-PC (λex 495 nm and λem 505 nm) and Hoechst 33342 (λex 352 nm and λem 461 nm) were excited at 543, 488 and 405 nm, respectively. Images were obtained using 20× and 100× lenses.

Results and discussion

Size of all liposomes under study was in the range of 85–90 nm as assessed by dynamic light scattering independently of the presence of SiaLe^X-PEG$_{8–15}$-DOG in the composition (Table 1). Low values of polydispersity indices, along with narrow peaks of size distribution (characterized by ''half-height half-width'' parameter), evidence homogeneity of the generated formulations. As for zeta potential, all

liposomes had similar negative charge values ~30 mV, which is due the relatively high content of PI (10 mol. %) in the bilayer. Slight shift of zeta potential to more negative values for the SiaLe^X-L formulation is explained by the lack of melphalan moiety, which at neutral pH is protonated at its primary amino group and thus positively charged, and is in agreement with our previous data [17].

Histology-based techniques remain a standard to assess antiangiogenic and/or antivascular properties of anticancer therapeutics [23,24]. In the case of the standard treatment protocol, haematoxylin and eosin (H&E) sections (Figure 2) demonstrated progressive changes in tumour morphology when passing from treatment with melphalan to liposomal Mlph-DOG and further to targeted prodrug-loaded liposomes. The effects characteristic of treatment with SiaLe^X-equipped liposomes (SiaLe^X-L-Mlph-DOG; Figure 2D) were the most pronounced. Vessel damage resulted in their dilation, plethora and escape of some erythrocyte into interstitial space. Some tumour areas combined apoptotic cell death and cell lysis with sharp decrease in the number of microvessels.

Table 1. Physical characteristics of the liposomes.

Liposome formulation	D (nm)	PDI	$H_{1/2}$ (nm)	ZP (mV)
L-Mlph-DOG	87.6 ± 0.2	0.052 ± 0.020	19.1 ± 3.8	-27.6 ± 1.1
SiaLe^X-L-Mlph	86.0 ± 1.6	0.074 ± 0.005	23.4 ± 1.2	-27.5 ± 1.5
SiaLe^X-L	88.0 ± 1.6	0.056 ± 0.014	20.5 ± 2.9	-33.1 ± 3.0

D, mean diameter; PDI, polydispersity index; $H_{1/2}$, half-height half-width; ZP, zeta potential. Mean ± SE of the measurements are presented.

Specific highlighting of tumour blood vessels with the CD31 endothelial cell marker (Table 2; Figure S1 in Supplementary Material) demonstrated that 1 week after the injections (day 15) both liposomal formulations of Mlph-DOG still caused considerable ($p < 0.01$ compared to PBS) decrease in the number of tumour vessels with SiaLe^X-L-Mlph producing greater antivascular effect than L-Mlph (72.3% growth inhibition against 29.4%). Also, statistically significant ($p < 0.01$) difference with Mlph was only observed in the case of SiaLe^X-L-Mlph formulation. Under delayed treatment protocol, the effect of considerable vascular disruption by liposomal formulations of Mlph-DOG was reproduced. After treatment with L-Mlph or SiaLe^X-L-Mlph, the integral number of vessels decreased as compared to Mlph. Large (>50 μm) dilated vessels with apoptotic endothelial cells in lumens appeared (Table 2; Figure 3D and E), which is further supported by TUNEL-positive apoptotic

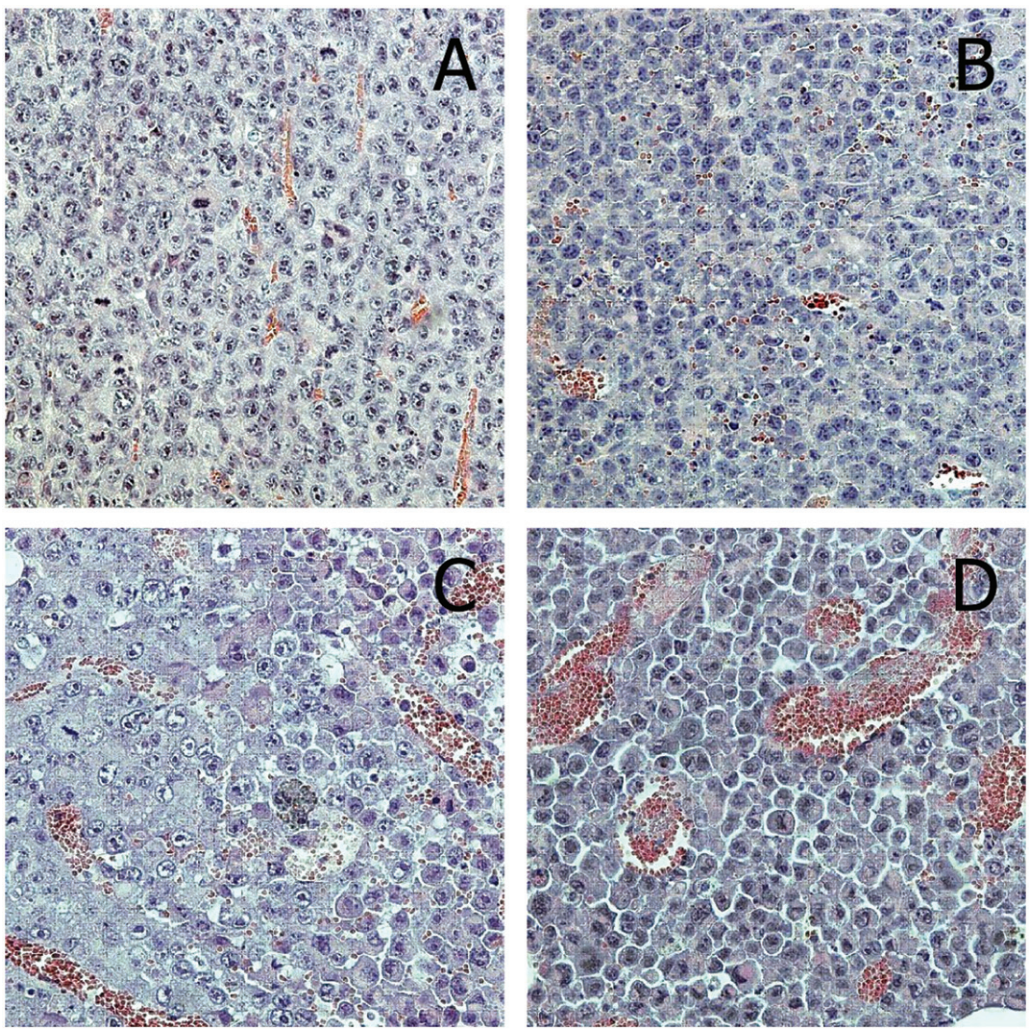

Figure 2. H&E staining of the Lewis lung carcinoma 5-μm sections excised on day 9 of the experiment after mice had received two intratail injections (on days 3 and 7) of (A) PBS, (B) 7 mg/kg melphalan and equivalent doses of (C) L-Mlph-DOG or (D) SiaLe^X-L-Mlph-DOG. (A) Short segments of microvessels filled with few erythrocytes are present in thick tumour tissue sections of control animals. (B) In melphalan-treated tumours, insignificant haemorrhages with erythrocytes escaping circulation are observed. Treatment with liposomes resulted in necrotic patches and significant haemorrhages (C and D) or even stagnant blood lakes in the case of the targeted formulation (D).

cells highlighted in the injured vessels of tumours treated with Mlph-DOG liposomal formulations (Figure S2 and comments). Targeted cytotoxic liposomes surpassed SiaLe^X-free ones ($p = 0.058$) as vessel disrupting agents and were the only ones to advance intact melphalan ($p < 0.001$) (Table 2).

Lycopersicon esculentum lectin (LEL) binds *N*-acetylglucosamine oligosaccharides exposed in abundance on the apical surface of endothelial cells. Upon intravenous injection, LEL can only reach its vascular target through circulation, allowing for visualization of functional tumour vessels [25]. Under the early treatment protocol, 2 days after the treatment, LEL-stained vessels in the sections were so few, no reliable comparison between different treatment groups could be made (data not shown). By day 15, both ligand-free and targeted liposome formulations produced considerable vessel damage (Table 3). However, the number of vessels accessible to LEL actually counted in the sections from liposome-treated groups were at the lower detection limit (7 ± 3 vessels per field of vision), which did not allow for comparison between the two liposome groups.

Table 2. Number of CD31-positive vessels per tumour section after two injections under standard and delayed treatment regimens.

	Microvessels, mean ± SD		
Group ($n = 3$)	Standard	Delayed	Large vessels*
Control (PBS)	23.8 ± 8.4	24.0 ± 2.9	3
SiaLe^X-L	–	20.5 ± 2.5	12
Melphalan	16.8 ± 4.9	18.4 ± 0.8¶	15
L-Mlph-DOG	9.4 ± 1.9#	14.9 ± 2.7¶	30
SiaLe^X-L-Mlph-DOG	6.6 ± 1.9##	10.2 ± 1.5¶¶	54

For each group (see caption to Figure 2), tumours of three animals were studied, two sections of each tumour were analyzed, five fields of vision in each. To assess statistical significance, two-tailed unpaired Student's *t*-test was used.

*For convenience, total number of large (>50 μm in diameter) dilated vessels produced under delayed treatment per 30 observations is reported; as for the number of vessels per tumour section, significant differences ($p < 0.05$) were observed between control (PBS) and all other groups except for SiaLe^X-L.

#$p < 0.01$, compared to control group (PBS).

##$p < 0.01$, compared to control (PBS) and melphalan groups and $p < 0.05$, compared to L-Mlph-DOG.

¶$p < 0.05$, compared to control group (PBS).

¶¶$p < 0.005$, compared to control (PBS), SiaLe^X-L and Mlph groups.

Table 3. Average number of functional vessels per tumour section after two injections under standard and delayed treatment regimens as assessed by intravital LEL perfusion.

	Number of perfused vessels, mean ± SD	
Group ($n = 3$)	Standard	Delayed
Control (PBS)	11.3 ± 2.4	12.5 ± 2.5
SiaLe^X-L	–	13.5 ± 1.1
Melphalan	11.3 ± 3.3	12.8 ± 3.2
L-Mlph-DOG	6.8 ± 2.6*	9.5 ± 0.1
SiaLe^X-L-Mlph-DOG	7.0 ± 3.4*	6.4 ± 0.9**

For each group, tumours of three animals were studied, two sections of each tumour were analyzed, five fields of vision in each. To assess statistical significance two-tailed unpaired Student's *t*-test was used.

*$p < 0.05$, compared to control (PBS) and Mlph groups.

**$p < 0.05$, compared to control (PBS), SiaLe^X-L and Mlph groups, as well as between the two liposome formulations, L-Mlph-DOG and SiaLe^X-L-Mlph-DOG.

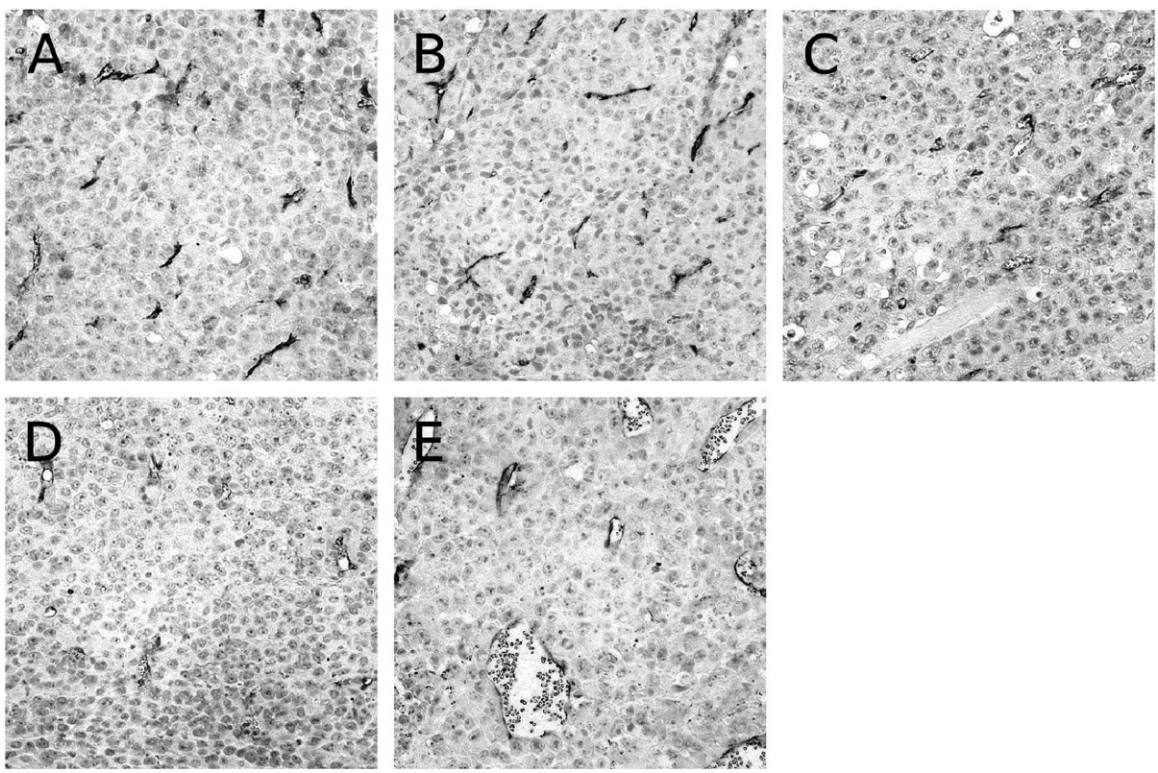

Figure 3. CD31 expression in tumours from mice treated twice (days 7 and 10) with PBS (A), drug-free SiaLe^X-liposomes (B), Mlph at dose 7 mg/kg (C), and equivalent doses of liposomal Mlph-DOG (D) or SiaLe^X-liposomes with Mlph-DOG (E) on day 12 after Lewis lung carcinoma transplantation (delayed treatment protocol). (A and B) Multiple microvessels with lumens are present in histological sections of control mice. (C) Treatment with melphalan notably decreased vessel quantity. (D and E) In tumours treated with liposomes, only sparse fragments of microvessels are observed, their number being especially low in the case of SiaLe^X-liposomes; large dilated vessels appear instead (E). Representative pictures for each experimental group are presented.

When treated later in the development (delayed protocol), tumours possess clearly developed and still growing vasculature. Delayed regiment of treatment proved beneficial, resulting in substantial vascular disruption caused by liposome formulations as compared to PBS and melphalan (Figure S3), as well as statistically significant difference between the $SiaLe^X$-free and targeted formulation: the latter induced a decrease in the number of viable vessels by ~33% as compared to $SiaLe^X$-free formulation (Table 3).

The fact that non-targeted liposome formulations caused nearly as severe damage to tumour vessels as did their $SiaLe^X$-targeted counterparts (under standard treatment protocol) may be due to the absence of preferential accumulation of $SiaLe^X$-liposomes in tumour over the non-targeted ones. At tumour site, endothelial cells are probably the primary targets of liposomes of either kind, as observed in H&E sections (Figure 2C and D). Then, non-targeted liposomes are free to extravasate inside tumour tissue and exert their effect against tumour cells, while targeted liposomes are expected to take over a different route to cross the endothelial barrier. Presumably, the multivalent selectin–$SiaLe^X$ interactions keep them associated with endothelial cells longer, which results in the strongest disrupting effect on the vessels (Figure 2D). Indeed, fluorescently labelled $SiaLe^X$–targeted liposomes designed by Hirai et al. were observed shifted from blood to the surrounding tissues at 48 h after injection [26].

Slower recovery of CD31-positive vessels a week after treatment could be attributed to delayed antiangiogenic effect of $SiaLe^X$-liposomes due to, hypothetically, toxicity of $SiaLe^X$-targeted liposomal carrier or inhibition of selectin ligand-binding sites impairing the angiogenesis signalling routes. To exclude the possibility, a control group of Mlph-DOG-free liposomes bearing $SiaLe^X$-conjugate (sample $SiaLe^X$-L) was included in the delayed-protocol experiment. As assessed by intravital perfusion with LEL and anti-CD31 staining, injections of targeted non-cytotoxic liposomes did not affect vessel functionality (Table 3) and caused minor, if any, effect on vessel integrity (Table 2), which discredited the hypothesis.

Intratumoural localization of fluorescently labelled liposomes monitored by the analysis of tumour cryosections on day 7 after tumour transplantation coheres with CD31 and H&E data. Administration of BODIPY-PC-labelled $SiaLe^X$-liposomes to untreated mice led to co-localization of their fluorescence (green) with CD31 fluorescent marker (red) of vessels, while non-targeted

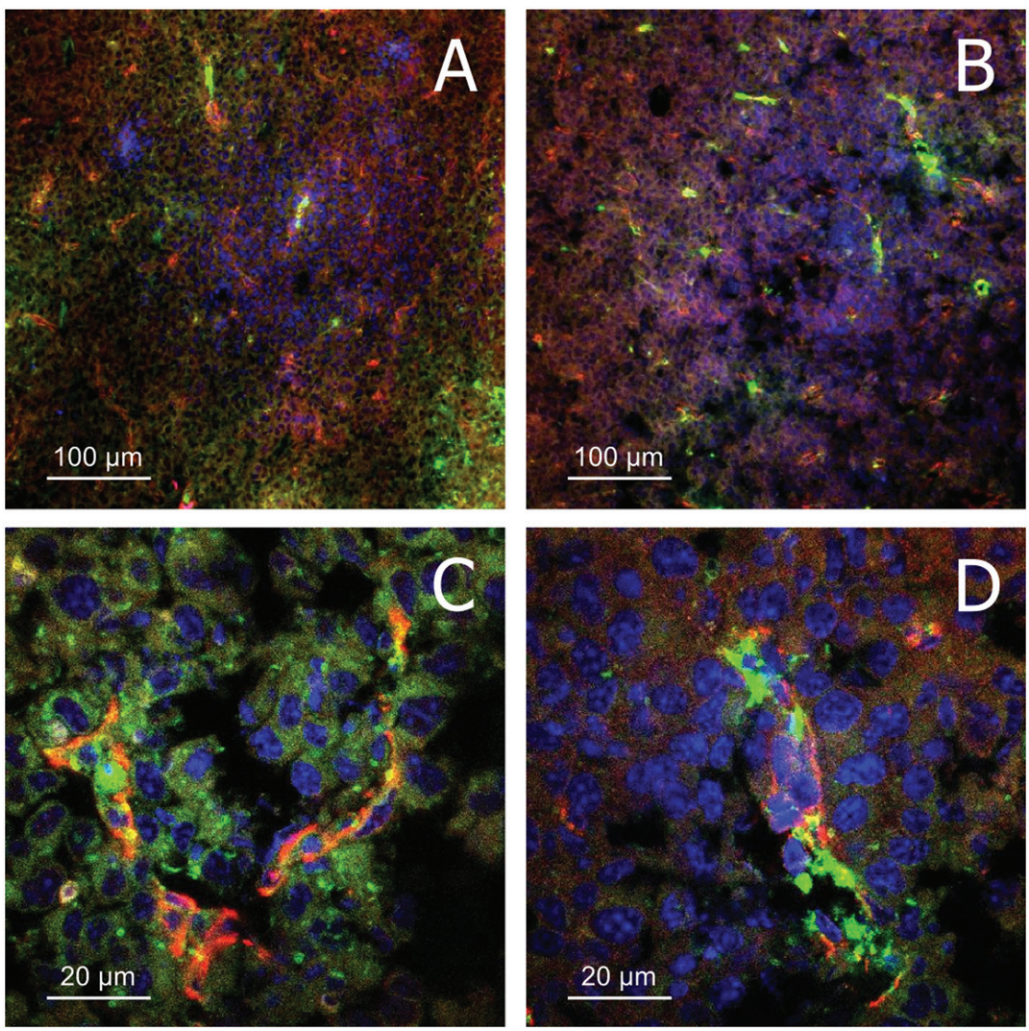

Figure 4. Confocal laser scanning microscopy images of tumour cryosections obtained from untreated mice administered with BODIPY-PC-labelled (A and C) non-targeted and (B and D) $SiaLe^X$-targeted liposome formulations loaded with Mlph-DOG prodrug, on day 7 after tumour transplantation. Typical fields of vision selected in the course of the analysis are presented. Hoechst staining of the nuclei (blue) was produced upon intravenous injection of 15 mg/mL Hoechst solution 20 min after liposome administration. Sections were immunostained with CD31 antibodies (visualized with secondary Alexa conjugated IgG, red). Non-targeted liposomes exhibit a diffuse pattern of BODIPY-PC fluorescence (green) within the tumour tissue (A and C). Circular and longitudinal distribution patterns of the fluorescence of $SiaLe^X$-liposomes associate with blood vessels (B and D).

formulation distributed over tumour tissue and produced diffuse fluorescence in perivascular regions (Figure 4). Thus, the SiaLe^X-conjugate confines/directs liposomes to the angiogenic endothelium.

There is a growing body of evidence that it is the higher uptake in tumour (or endothelial) cells rather than accumulation at tumour tissue that ensures higher performance of a variety of targeted nano-sized delivery systems, as discussed by Phillips and co-authors ([27], and references therein). We assume that upon binding endothelial cells, SiaLe^X-liposomes undergo internalization resulting in cell disruption due to the cytotoxic alkylating action of melphalan generated from the prodrug intracellularly. A fraction of SiaLe^X-liposomes may also bind to E-selectin on tumour endothelium and then enter gaps between the endothelial cells – similar to the way it is speculated for the cisplatin-loaded SiaLe^X-conjugated liposomes (though of a different, more sophisticated lipid composition, also coated with human serum albumin to prevent opsonization) [28]. The latter ones also exemplify moderate, yet reliable gain in tumour growth inhibition over SiaLe^X-free formulation in a mouse xenograft model of A549 lung carcinoma [28].

Antitumour effect of Mlph-DOG liposomal formulations upon early administration (standard protocol) surpassed melphalan mildly, adding only 14–29% (days 14–21) to the rate of tumour growth inhibition by the intact drug (Figure 5A); no statistically significant difference between liposomes of two types was observed. Towards the end of the experiment, however, only SiaLe^X-targeted cytotoxic liposomes retained the advantage over melphalan ($p < 0.024$, Figure 5A). Somewhat different results were obtained under the delayed regimen of treatment. Non-targeted Mlph-DOG liposomes inhibited tumour growth by 10–35% (days 20–24) as compared to intact melphalan, while the increment for SiaLe^X-L-Mlph-DOG liposomes was 17–47% (Figure 5B). By the end of the delayed-protocol experiment targeted formulation not only differed significantly from the intact drug ($p < 0.015$) (Figure 5B), but demonstrated a tendency to inhibit tumour growth more effectively than the non-targeted one.

Non-cytotoxic SiaLe^X-L formulation did not exert any effect on growth of tumours (Figure 5B), which agrees with it being inert vascular disrupting agent (Tables 2 and 3) and additionally argues in favour of the apoptotic mechanism of cytotoxic antivascular action of the targeted Mlph-DOG formulation.

Thus, the observed antivascular effect of cytotoxic SiaLe^X-liposomes did not provide for substantial reinforced antitumour effect. A different treatment protocol, e.g. multiple dosing, might potentiate the targeted therapy. Meanwhile, design of a delivery system for melphalan – a cell-cycle non-specific alkylating agent – that would combat its severe side effects, is of high demand. Melphalan alone is still indispensable for treating late stages of diseases and metastasizing malignancies [29]; melphalan-with-prednisone has been the standard chemotherapy for multiple myeloma for over 40 years and continues to be the core of many combination therapy regimens [29,30]. To our knowledge, the attempts to efficiently encapsulate melphalan *per se* in a nanocarrier system were not successful [31]. Targeted SiaLe^X-liposomes bearing melphalan lipophilic

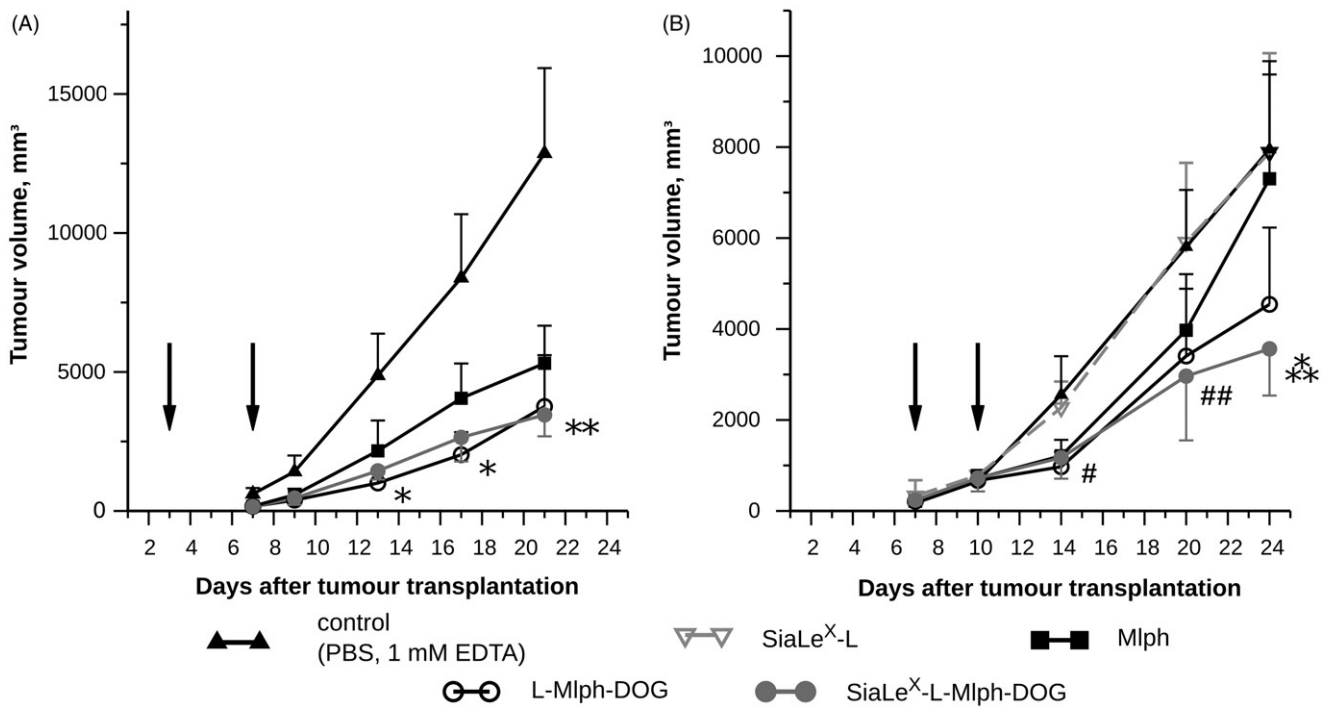

Figure 5. Lewis lung carcinoma growth dynamics in mice upon two injections (indicated with arrows) of the preparations under standard (on days 3 and 7) and delayed (on days 7 and 10) treatment regimens. (*) significant ($p < 0.03$) differences between melphalan and L-Mlph-DOG groups; (**) significant ($p < 0.03$) differences between melphalan and SiaLe^X-L-Mlph-DOG groups; (#) significant differences between control and L-Mlph-DOG ($p < 0.001$) or SiaLe^X-L-Mlph-DOG ($p < 0.01$) groups; (##) significant differences between control and both liposome groups L-Mlph-DOG and SiaLe^X-L-Mlph-DOG ($p < 0.01$) and (***) significant differences between control and L-Mlph-DOG ($p < 0.005$), control and SiaLe^X-L-Mlph-DOG ($p < 0.001$), Mlph and L-Mlph-DOG ($p < 0.05$) and Mlph and SiaLe^X-L-Mlph-DOG ($p < 0.03$).

prodrug, proven haemocompatible in an *in vitro* test panel [17], are definitely a candidate formulation not only to alleviate melphalan-associated toxicity, but also to extend the list of indications for the drug.

Conclusions

Our present findings supplement the still scarce knowledge on $SiaLe^X$-mediated effects *in vivo*. For the first time, anti-inflammatory cardioprotective effect of $SiaLe^X$-conjugated long-circulating PEG-liposomes as such was shown in a feline model [32]; then, similar liposomes were shown to inhibit E-selectin mediated cell adhesion [33] and tumour cell adhesion to vascular endothelium *in vitro* [34]. Hirai and co-workers provided evidence that liposomes equipped with $SiaLe^X$ ligand can target inflammatory and tumour sites *in vivo* [26]. Here, we demonstrate that $SiaLe^X$-liposomes, of a different composition, loaded with a cytotoxic lipophilic prodrug in the bilayer, target tumour vasculature *in vivo* and cause antivascular effect.

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Declaration of interest

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References

1. Lopes de Menezes DE, Pilarski LM, Allen TM. In vitro and in vivo targeting of immunoliposomal doxorubicin to human B-cell lymphoma. Cancer Res 1998;58:3320–30.
2. Schiffelers R, Storm G. Liposomal nanomedicines as anticancer therapeutics: beyond targeting tumor cells. Int J Pharm 2008;364: 258–64.
3. Fens MHAM, Hill KJ, Issa J, et al. Liposomal encapsulation enhances the antitumour efficacy of the vascular disrupting agent ZD6126 in murine B16.F10 melanoma. Br J Cancer 2008;99: 1256–64.
4. Vader P, Crielaard BJ, Van Dommelen SM, et al. Targeted delivery of small interfering RNA to angiogenic endothelial cells with liposome-polycation-DNA particles. J Control Release 2012;160: 211–6.
5. Wicki A, Rochlitz C, Orleth A, et al. Targeting tumor-associated endothelial cells: anti-VEGFR2 immunoliposomes mediate tumor vessel disruption and inhibit tumor growth. Clin Cancer Res 2012; 18:454–64.
6. Ehrhardt C, Kneuer C, Bakowsky U. Selectins – an emerging target for drug delivery. Adv Drug Deliv Rev 2004;56:527–49.
7. Witz IP. The selectin-selectin ligand axis in tumor progression. Cancer Metastasis Rev 2008;27:19–30.
8. Barthel S, Gavino J, Descheny L, Dimitroff C. Targeting selectins and selectin ligands in inflammation and cancer. Expert Opin Ther Targets 2007;11:1473–91.
9. Köhler S, Ullrich S, Richter U, Schumacher U. E-/P-selectins and colon carcinoma metastasis: first in vivo evidence for their crucial role in a clinically relevant model of spontaneous metastasis formation in the lung. Br J Cancer 2010;102:602–9.
10. Jubeli E, Moine L, Vergnaud-Gauduchon J, Barratt G. E-selectin as a target for drug delivery and molecular imaging. J Control Release 2012;158:194–206.
11. Liu ZJ, Tian R, Li Y, et al. Inhibition of tumor angiogenesis and melanoma growth by targeting vascular E-selectin. Ann Surg 2011; 254:450–6.
12. Foxall C, Watson SR, Dowbenko D, et al. The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis(x) oligosaccharide. J Cell Biol 1992;117: 895–902.
13. Vodovozova EL, Moiseeva EV, Grechko GK, et al. Antitumour activity of cytotoxic liposomes equipped with selectin ligand SiaLe(X), in a mouse mammary adenocarcinoma model. Eur J Cancer 2000;36:942–9.
14. Kuznetsova N, Kandyba A, Vostrov I, et al. Liposomes loaded with lipophilic prodrugs of methotrexate and melphalan as convenient drug delivery vehicles. J Drug Deliv Sci Tech 2009; 19:51–9.
15. Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc Natl Acad Sci USA 1988;85:6949–53.
16. Peng A, Straubinger RM, Balu-Iyer SV. Phosphatidylinositol containing lipidic particles reduces immunogenicity and catabolism of factor VIII in hemophilia a mice. AAPS J 2010;12:473–81.
17. Kuznetsova NR, Sevrin C, Lespineux D, et al. Hemocompatibility of liposomes loaded with lipophilic prodrugs of methotrexate and melphalan in the lipid bilayer. J Control Release 2012;160: 394–400.
18. Vodovozova EL, Pazynina GV, Bovin NV. Synthesis of diglyceride conjugate of selectin ligand $SiaLe^X$ as a vector for targeting of drug-loaded liposomes. Mendeleev Commun 2011;21: 69–71.
19. Vodovozova EL, Nikol'skii PI, Mikhalev II, Molotkovskii IG. Lipid derivatives of sarcolysin, methotrexate and rubomycin. Russ J Bioorg Chem 1996;22:468–75.
20. Boldyrev IA, Zhai X, Momsen MM, et al. New BODIPY lipid probes for fluorescence studies of membranes. J Lipid Res 2007;48: 1518–32.
21. Khromova NV, Kopnin PB, Stepanova EV, et al. p53 hot-spot mutants increase tumor vascularization via ROS-mediated activation of the HIF1/VEGF-A pathway. Cancer Lett 2009;276: 143–51.
22. Tsimafeyeu I, Zaveleva E, Stepanova E, Low W. OM-RCA-01, a novel humanized monoclonal antibody targeting fibroblast growth factor receptor 1, in renal cell carcinoma model. Invest New Drugs 2013;31:1436–43.
23. Wang D, Stockard CR, Harkins L, et al. Immunohistochemistry in the evaluation of neovascularization in tumor xenografts. Biotech Histochem 2008;83:179–89.
24. Ullrich RT, Jikeli JF, Diedenhofen M, et al. In-vivo visualization of tumor microvessel density and response to anti-angiogenic treatment by high resolution MRI in mice. PLoS One 2011;6: e19592.
25. Hashizume H, Baluk P, Morikawa S, et al. Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol 2000;156:1363–80.
26. Hirai M, Minematsu H, Kondo N, et al. Accumulation of liposome with Sialyl Lewis X to inflammation and tumor region: application to in vivo bio-imaging. Biochem Biophys Res Commun 2007;353: 553–8.
27. Phillips M, Gran M, Peppas N. Targeted nanodelivery of drugs and diagnostics. Nano Today 2010;5:143–59.
28. Hirai M, Minematsu H, Hiramatsu Y, et al. Novel and simple loading procedure of cisplatin into liposomes and targeting tumor endothelial cells. Int J Pharm 2010;391:274–83.
29. Musto P, D'Auria F. Melphalan: old and new uses of a still master drug for multiple myeloma. Expert Opin Investig Drugs 2007;16: 1467–87.
30. Mateos MV, San-Miguel J. Treatment of newly diagnosed myeloma in patients not eligible for transplantation. Curr Hematol Malig Rep 2011;6:113–19.
31. Krasnov VP, Korolyova MA, Vodovozova EL. Nano-sized melphalan and sarcolysin drug delivery systems: synthesis and prospects of application. Russ Chem Rev 2013;82:783–814.
32. Murohara T, Margiotta J, Phillips LM, et al. Cardioprotection by liposome-conjugated sialyl Lewis x-oligosaccharide in myocardial ischaemia and reperfusion injury. Cardiovasc Res 1995;30: 965–74.

33. DeFrees SA, Phillips L, Guo L, Zalipsky S. Sialyl Lewis x liposomes as a multivalent ligand and inhibitor of E-selectin mediated cellular adhesion. J Am Chem Soc 1996;118: 6101–4.

34. Zeisig R, Stahn R, Wenzel K, et al. Effect of sialyl Lewis X-glycoliposomes on the inhibition of E-selectin-mediated tumour cell adhesion *in vitro*. Biochim Biophys Acta 2004; 1660:31–40.

Supplementary material available online

Supplementary Figures S1–S3