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ORIGINAL ARTICLE

Experimental Anticancer Therapy with Vascular-disruptive Peptide and Liposome-entrapped Chemotherapeutic Agent

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Abstract Vasculature is essential for the sustained growth of solid tumors and metastases. Tumor cells surviving vascular-disruptive therapeutic intervention (especially those present at the tumor rim) can contribute to tumor regrowth. The aim was to strengthen, by carrier-mediated delivery of a chemotherapeutic, the curative effects of a bifunctional antivascular oligopeptide capable of inducing vascular shutdown and tumor shrinkage. For the in vitro experiments and animal therapy, ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ peptide (900 μM in D-PBSA, i.e. Dulbecco's PBS without Ca^{2+} and Mg^{2+}) and size-calibrated, passively or actively targeted liposomes based on distearoylphosphatidylcholine, cholesterol, and *N*-carbamoyl-methoxypolyethyleneglycol coupled to distearoylphosphatidylethanolamine (PEG–DSPE) and containing gradient-entrapped doxorubicin were used. The KB (human nasopharyngeal carcinoma) cell line overexpressing folate receptors was used in the fluorescence studies of liposomal uptake. The B16-F10 melanoma cell line was used for confirming, by flow cytometry and confocal microscopy, doxorubicin intracellular transfer as well as to induce experimental tumors in C57BL/6 mice. Animal therapy was achieved with injections of vascular-disrupting peptide, doxorubicin-loaded liposomes, or alternating combined therapy. The results (tumor growth inhibition and survival) were compared using the Mann–Whitney *U* test and the log-rank test. Necrosis in H&E-stained tumor sections was assessed microscopically by pathologists. Treatment of C57BL/6 mice bearing B16-F10 experimental tumors with a combination of vascular-disruptive peptide and doxorubicin-carrying pegylated liposomes (either passively targeted liposomes (PTL) or folate receptor targeted) gave better therapeutic effects when tumor development was re-challenged with a second cycle of combined therapy. Marked inhibition of tumor growth and a statistically significant extension of the lifespan of the treated mice were observed when the re-challenge involved the use of folate receptor-targeted liposomes (FTL). Anticancer therapy involving vascular-disruptive peptide and doxorubicin delivered via pegylated folate receptor-targeted liposomes is more effective than either monotherapy, especially when tumor growth is re-challenged with the therapeutic combination.

Keywords Vascular-disruptive agents · Pegylated liposomes · Doxorubicin · Combined antitumor therapy

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Introduction

The growth of the majority of tumors depends on the formation of blood supply (Folkman 1990, 1996; Kerbel and Folkman 2002). Anticancer therapeutic strategies should therefore possibly target the tumor neovasculature. These

so-called antiangiogenic strategies have been described in numerous articles (see, for example, Kerbel and Folkman 2002; Scappaticci 2002; Verheul et al. 2004). Several antiangiogenic drugs have been clinically evaluated (Dickson et al. 2007; Kerbel 2000, 2001; Ma and Waxman 2009; Motzer et al. 2007). They seem to be best suited for treating early-stage or metastatic disease (Horsman and Siemann 2006).

Denekamp proposed another strategic approach: blood vessel-reactive therapy that targets preexisting tumor vasculature (Denekamp 1984, 1993). Although the concept is not new, it has only recently emerged as a therapeutic possibility, especially for the treatment of bulky disease (Siemann and Rojiani 2005). The vascular-disruptive therapeutic strategy is based on two types of drugs (Siemann et al. 2004; Szala 2004; Thorpe 2004). The first are low-molecular-weight drugs, such as combretastatin and its derivatives, acting mainly upon cytoskeletal tubulins in tumor endothelial cells (Tozer et al. 2002). The second type of vascular-damaging agents are high-molecular-weight drugs with two domains: one recognizing endothelial cell surface markers (recognition domain) and the other destroying the targeted cell (effector domain). Recognition domains can be formed by ligands recognizing $\alpha_v\beta_3$ integrin receptors on tumor endothelial cell surface (Arap et al. 1998; Ellerby et al. 1999; Hood and Cheresh 2002). Such receptors must be internalized so that the effector domain of the antivascular agent can reach the cell interior (Allen 2002). Effector domains of vascular-disruptive drugs are coagulation-inducing proteins, cytostatics, radioisotopes, cytokines, and proapoptotic oligopeptides (Siemann et al. 2004; Thorpe 2004).

In this study we sought to improve the antitumor effectiveness of a two-domain antivascular oligopeptide, ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ (Ellerby et al. 1999) by using it in combination with a proven chemotherapeutic. The oligopeptide is highly toxic to $\alpha_V\beta_3$ integrin receptor-expressing endothelial cells as well as to B16-F10 murine melanoma cells. The effects induced by this compound were the subject of our earlier study wherein monotherapy of melanoma tumor-bearing C57BL/6 mice with the peptide inhibited tumor growth, but failed to extend animal survival (Smolarczyk et al. 2006).

The rationale for trying to improve the antitumor effect of the peptide in question by adding chemotherapy was based on the observation that the effect of vascular-disruptive agents is typically restricted to the central part of the tumor, which leaves a rim of viable cells at the tumor's periphery (Horsman and Siemann 2006). Tumor rim cells receive their nutritional support from neighboring normal blood vessels, which would not be affected by the use of the examined oligopeptide. Thus, we administered the peptide in combination with doxorubicin entrapped inside long-lived liposomes. This chemotherapeutic is a well-known anthracycline drug active against various neoplasms (Soloman and Gabizon 2008). Doxorubicin is also expected to exert an antiangiogenic effect on tumor endothelium (Browder et al. 2000). To diminish doxorubicin's toxicity it was entrapped in pegylated liposomes (PTL) composed of distearoylphosphatidylcholine, cholesterol and *N*-carbamoyl-methoxypolyethyleneglycol coupled to distearoylphosphatidylethanolamine (PEG–DSPE). The PEG layer that forms the outer shield in liposomes imparts resistance against opsonization and extends the drug carrier's lifespan in the circulation (Gabizon et al. 2002). Liposomal shielding of the drug would also allow bypassing the multidrug resistance (MDR) of the examined B16-F10 melanoma cells (Levine et al. 2004). Long-lived liposomes are capable of passively targeting solid neoplasms because of leaky tumor vasculature (e.g. Huwyler et al. 2008; Maeda 2001). In order to target neoplastic cells of the tumor periphery and strengthen a possibly additive therapeutic effect of the peptide–chemotherapeutic combination, we employed doxorubicin-loaded PTL with attached folate ligands. Malignant cells of epithelial origin are thought to express folate receptors abundantly on their surface (e.g. Gabizon et al. 1999).

Materials and Methods

Cell Cultures

The B16-F10 melanoma cell line (ATCC, Manassas, VA, USA) used to induce experimental tumors in mice was maintained in RMPI 1640 medium (Gibco, USA) supplemented with 10% FBS (MP Biomedicals, USA) and antibiotics (1:100 penicillin–streptomycin). Cultures were grown in T25 flasks (Corning, USA) kept in a standard 37°C incubator in a humidified atmosphere containing 5% CO_2 (Eliaz and Szoka 2001, with modifications). Cultures of B16-F10 cells were trypsinized and the cells were collected immediately before inoculating the animals (Mitrus et al. 2006; Smolarczyk et al. 2006). The KB cell line (Institute of Immunology and Experimental Therapy, Wroclaw, Poland), derived from human nasopharynx carcinoma and used in the studies of folate receptor-mediated uptake, was grown in serum-supplemented folate-free RPMI 1640 medium (Gibco, USA); 24 h prior to the experiments, this culture medium was switched to one without serum (Lee and Low 1995).

Therapeutic Oligopeptide

The peptide used in the experimental therapy, ACD CRGDCFC-GG-$_D$(KLAKLAK)$_2$ (further referred to as

RGD-KLAKLAK), was originally designed by Ellerby et al. (1999). The batch used in this study was synthesized by GeneScript (Piscataway, NJ, USA). The peptide solution (in D-PBSA) used for injections was 900 μM (Smolarczyk et al. 2006).

Preparation of Liposomes

Passively targeted liposomes (PTL) were prepared at a 9:5:1 [mol/mol] ratio from distearoylphosphatidylcholine, cholesterol, and *N*-carbamoyl-methoxypoly ethyleneglycol coupled to distearoylphosphatidylethanolamine (DSPE–PEG) (Zhou et al. 2002). To form folate receptor-targeted liposomes (FTL), *N*-carbamoyl-methoxypolyethyleneglycol conjugated with folate (DSPE–PEG–folate) was added at 0.1 mol%. The phospholipids and cholesterol were from Avanti Polar Lipids (Alabaster, AL, USA). The liposomes were formed by solvating chloroform-dried lipid mixture films with 250 mM ammonium sulfate (pH 5.5), freeze–thawing (5×) the emulsions, and extrusion at above the gel–liquid crystal transition temperature of lipid components (65°C) through two stacked 100-nm polycarbonate membranes. The liposomes were then dialyzed (Spectrum membrane, 10,000 molecular weight cut-off, MWCO) in isotonic sucrose (10%). Doxorubicin (Sigma, USA) was entrapped inside the liposomes at a 0.1:1 (w/w) drug/lipid ratio (in excess of 80%, as judged from absorbance measurements (490 nm) of acidic methanol-solubilized liposomes and assuming $\varepsilon_a = 12{,}500\ M^{-1}\ cm^{-1}$) using a proton-gradient procedure (Madden et al. 1990; Sharma et al. 1997) with minor modifications. The phospholipid concentration was confirmed by phosphorus assay using ammonium molybdate and Fiske–Subbarow reagent (Bartlett 1959). The optical density of the samples at 830 nm was recorded spectrophotometrically. The liposomes were sterilized through 0.2-μm filters (Millipore, USA) and stored in the dark at 4°C until use.

Liposomes encapsulating calcein were prepared as outlined above with the exception of solvating the lipid film with 25 mM calcein solution in D-PBSA (Lee and Low 1995).

Liposome Size Measurements

The size and size distribution of the liposomes were determined by photon correlation spectroscopy using a dynamic light-scattering particle-size analyzer (Zetasizer Nano model, Malvern, UK) (Abraham et al. 2005). The analysis indicated a discrete mono-modal log-normal size distribution in the range between 70 and 200 nm in diameter, with a mean diameter of liposomes not exceeding 130 nm.

Liposome-mediated Transfer In Vitro and Intracellular Localization of Doxorubicin

Doxorubicin transfer using the liposome carriers into the B16-F10 cells cultured in vitro was confirmed by flow cytometry and confocal microscopy (Goren et al. 2000; with minor modifications). In brief, for FACS analysis the liposomal emulsions (final doxorubicin concentration: 100 μg/ml) were diluted with Opti-MEM and added to ca. 50% confluent B16-F10 cultures, which were then incubated at 37°C in 5% CO_2 for 4 h. Doxorubicin fluorescence associated with the fraction of liposome-transfected cells was analyzed using D-PBSA-washed and trypsinized cell cultures resuspended in D-PBSA and a FACSCanto instrument (BD Biosciences, NJ, USA; 20 mW air-cooled blue laser source; emission line $\lambda_{ex} = 488$ nm; emission filter $\lambda_{em} = 550$ nm). To visualize the intracellular accumulation of doxorubicin by confocal microscopy, B16-F10 cells growing on a coverglass (Nunc, USA) in 3-cm plastic dishes were transfected the same as for FACS and incubated for a further 18 h. Images of the untrypsinized cell cultures were generated using an LSM 510 confocal laser scanning microscope (Carl Zeiss GmbH, Germany). The images were stored in TIF format and analyzed using KS400 software (Carl Zeiss GmbH, Germany).

Uptake of PTL and FTL Liposomes by KB Cells

Liposomes entrapping membrane-impermeant calcein (10 mM concentration) were added to KB cells (ca. 5×10^5) growing in 33-mm dishes. The cultures were then kept in an incubator for varying times at either 4 or 37°C. Then the cells were rinsed three times with cold D-PBSA and treated with lysis buffer containing 1% Triton X-100. Cell-associated fluorescence in the extracts was then quantified using a PerkinElmer (MA, USA) fluorescence spectrophotometer (Lee and Low 1994). The number of cells in the lysed samples was inferred from total cellular protein measurements (Bradford assay). The fraction of internalized liposomes was assessed by stripping the cells of surface-attached liposomes by three washes with acidic D-PBSA (pH 3) and one final wash with D-PBSA (pH 7.2). The cell-attached liposomes that remained were assumed to be internalized.

Folate Receptor's Role in the Uptake of FTL Liposomes by B16-F10 and KB Cells

Folate receptor-targeted liposomes (60 nM) suspended in medium containing different concentrations of free folate were added to 5×10^5/cells (KB or B16-F10) cultured in 3-cm plastic dishes. The decrease in liposome binding was then measured as described above.

Animal Therapy with RGD-KLAK Oligopeptide and Doxorubicin-loaded Liposomes

All procedures involving animals were performed with the approval of the Local Ethics Committee, Silesian Medical University, Katowice, Poland. The therapy was performed using 6–8-week-old C57BL/6 female mice from our own animal facility. One week prior to the start of the experiments the mice used in studies involving the uptake of FTL were placed on a folate-free diet which continued until 3 days after the final therapeutic intervention. All mice used in the experiments were injected subcutaneously (dorsolateral flank) with B16-F10 melanoma cells (2×10^5/100 µl D-PBSA, pH 7.2). Mice with size-matched tumors were randomly assigned ($n = 5$) to different treatment groups and therapy was initiated when the tumors reached ca. 4 × 4 mm (on the 6th day post tumor inoculation). Mice in the control groups received either no treatment or vehicle-only treatment (D-PBSA as the control for therapeutic peptide or 10% sucrose-diluted empty liposomes as the control for liposomal doxorubicin). Mice in the experimental groups received either liposomal (PTL or FTL) doxorubicin treatment (intravenously) or antivascular peptide treatment (intratumorally) or the combined treatment (Lu et al. 2004 with modifications; Smolarczyk et al. 2006). Liposomal doxorubicin was administered on a schedule of 2 mg/kg delivered every other day for a week (one cycle or two cycles 10 days apart). The antivascular peptide was administered on a schedule of 12.5 mg/kg delivered every other day for a week (one cycle or two cycles 10 days apart). During the course of the combined therapy experiments, liposomal doxorubicin injections alternated with peptide injections (see below). The liposomal emulsions (containing no doxorubicin or 40 µg of the drug) were prepared by diluting liposomal stocks to 100 µl with 10% sucrose and administered to the mice's tail vein on the 6th day post tumor inoculation and every other day thereafter (four times in total). The antivascular peptide (250-µg aliquot of the drug) diluted to 100 µl with D-PBSA (pH 7.2), or just D-PBSA were administered intratumorally starting on the 7th day post tumor inoculation and every other day thereafter (four times in total). The amounts of doxorubicin or peptide delivered during such, essentially single, therapeutic session were thus 160 µg of the chemotherapeutic and 1 mg of the peptide. In some of the experiments this single cycle of injections was repeated following a 10-day interval. Mouse body weight was recorded and tumor size measured using calipers. Tumor growth rates (expressed as tumor volumes vs. time) were calculated from the formula: volume = length × width^2 × 0.52 and plotted. Survival of animals was monitored daily and the data served to generate a Kaplan–Meier plot.

Hematoxylin and Eosin Staining

C57BL/6 mice with experimentally induced B16-F10 tumors (for details see Animal Therapy section) were killed and the tumors were excised 24 h after a one-time administration of the therapeutic agents (doxorubicin-loaded PTL liposomes or antivascular peptide or PTL liposomes followed by the peptide on the following day). The tumor material was formalin fixed, paraffin embedded, and cut. The sections (6–8 µm thick) were H&E-stained and examined independently by two experienced pathologists. The extent of necrosis was computer analyzed with the help of our own algorithm.

Statistics

Statistical comparison of the tumor growth data was performed with the Mann–Whitney *U* test. Differences in the survival times of tumor-bearing animals were analyzed using the log-rank test. *p*-Values <0.05 were considered statistically significant.

Results and Discussion

Tumor vasculature-damaging agents can lead to the elimination of large portions of tumor, estimated to reach over 90% of tumor volume (Thorpe 2004). Despite this, viable neoplastic cells are invariably found at the tumor's periphery; such cells obtain oxygen and nutrients from the normal blood vasculature (e.g. Siemann et al. 2004). Tumor cells surviving therapy with vasculature-damaging agents are likely to contribute to tumor regrowth, making antivascular therapies only partially effective. It thus seemed worthwhile to attempt targeting the cell population that forms the tumor rim. Systemically delivered chemotherapeutics are convenient agents to achieve this goal (Horsman and Siemann 2006).

Our previous study (Smolarczyk et al. 2006) showed that the therapeutic peptide is indeed highly toxic for $\alpha_V\beta_3$-expressing endothelial cells as well as for B16-F10 cells (LC_{50} ca. 7 µM). We demonstrated that the peptide induced apoptosis in cultured B16-F10 cells. As $\alpha_V\beta_3$ integrin receptors are also found on B16-F10 cells, mice bearing B16-F10 experimental tumors seemed to offer a model well suited to the proposed combined treatment. Monotherapy with the peptide alone, although causing inhibited tumor growth, did not yield an extension of animal survival (Smolarczyk et al. 2006).

In the present study we investigated whether antitumor monotherapy of C57BL/6 mice bearing experimental tumors (B16-F10 malignant melanoma) with vascular-disruptive oligopeptide ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$

could be improved by combined use of this oligopeptide with a chemotherapeutic (doxorubicin) entrapped in liposomes that are sterically shielded with a PEG layer. Such pegylated (and presumably long-circulating) doxorubicin-loaded liposomes were approved for clinical use (Doxil, Caelyx) in various neoplastic conditions (breast and ovarian cancer, multiple myeloma, Kaposi sarcoma) because of their antitumor efficacy. The liposomal form of doxorubicin possesses important advantages over the free drug in terms of diminished cardiac toxicity and it allows overcoming excessive drug efflux linked to MDR-1 overexpression (Levine et al. 2004). Pegylated liposomal doxorubicin has an extended circulation time and has shown preferential accumulation in various implanted mouse–human tumors (Soloman and Gabizon 2008).

Since the size of liposomes is crucial for efficient extravasation at the tumor site, the extruded liposomal emulsions used in the therapeutic experiments were evaluated by photon correlation spectroscopy. The analysis demonstrated that the drug-loaded liposomes measured on average ca. 100 nm in diameter, the liposomes including folate ligand being slightly larger than those without the attached ligands. Since liposomal doxorubicin could be either released from the liposomes into the extracellular space or endocytosed with the liposomes by cancer cells, we investigated doxorubicin internalization by flow cytometry and confocal microscopy. Single peaks of doxorubicin fluorescence histograms (not shown) indicated that B16-F10 cells treated with doxorubicin-loaded liposomes efficiently internalized the drug. Confocal micrographs (not shown) confirmed a substantial intracellular presence of doxorubicin.

To verify the uptake of PTL and FTL liposomes by cells overexpressing folate receptors, KB (human nasopharyngeal cancer) cell cultures were exposed for 1–4 h at 37°C to liposomes encapsulating calcein, a membrane-impermeant dye. At the end of incubation, following extensive rinses with D-PBSA, the cell cultures were examined microscopically. Fluorescence due to FTL-mediated transfer, albeit limited initially to the cell periphery, was present after 4 h throughout the cytoplasm (Fig. 1a). The PTL liposomes also appeared to penetrate the examined cells, probably through endocytosis, although to a lesser extent than the FTL liposomes (Fig. 1b). When incubation was done at 4°C, only residual fluorescence was seen after 4 h, without an explicit difference between PTL- and FTL-treated cell cultures (not shown, but see Fig. 2b).

Intracellular fluorescence measurements demonstrated that the uptake of FTL liposomes was sigmoidal in shape and showed no further increase after ca. 4 h (Fig. 2a). This suggests some type of saturable uptake and internalization mechanism because during incubation the concentration of liposomes in the medium did not change significantly.

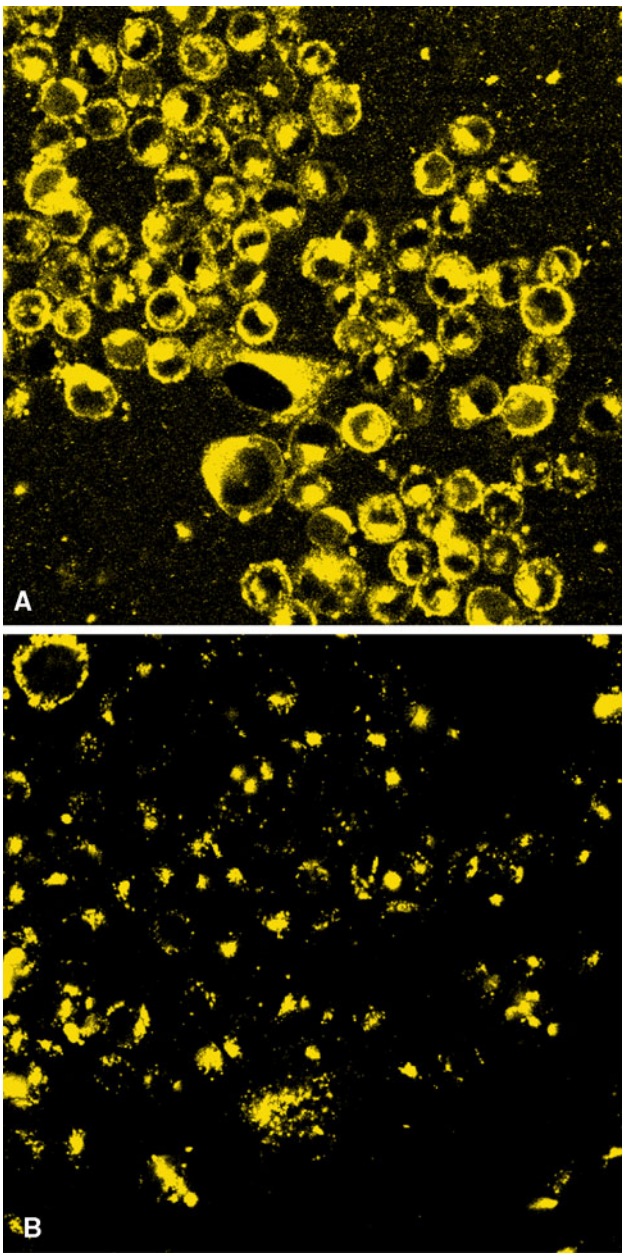

Fig. 1 Confocal micrographs of untrypsinized KB cell cultures exposed (4 h, 37°C) to calcein-loaded liposomes. Images show intense cytoplasmic fluorescence in (**a**) due to folate receptor-targeted liposomes (FTL), whereas that in (**b**), due to passively targeted liposomes (PTL), is weaker, but not negligible. Images were generated using a krypton–argon laser (488 nm line) and an LSM 510 confocal laser scanning microscope (Carl Zeiss GmbH)

To allow discriminating between cell surface-associated and endocytosed liposomes, the monolayers of KB cells were exposed to different amounts of FTL liposomes containing calcein and then subjected to differential rinses, either with D-PBSA to remove unbound liposomes or with acidic saline to strip uninternalized liposomes. Following lysis of the cell monolayers, the remaining fluorescence

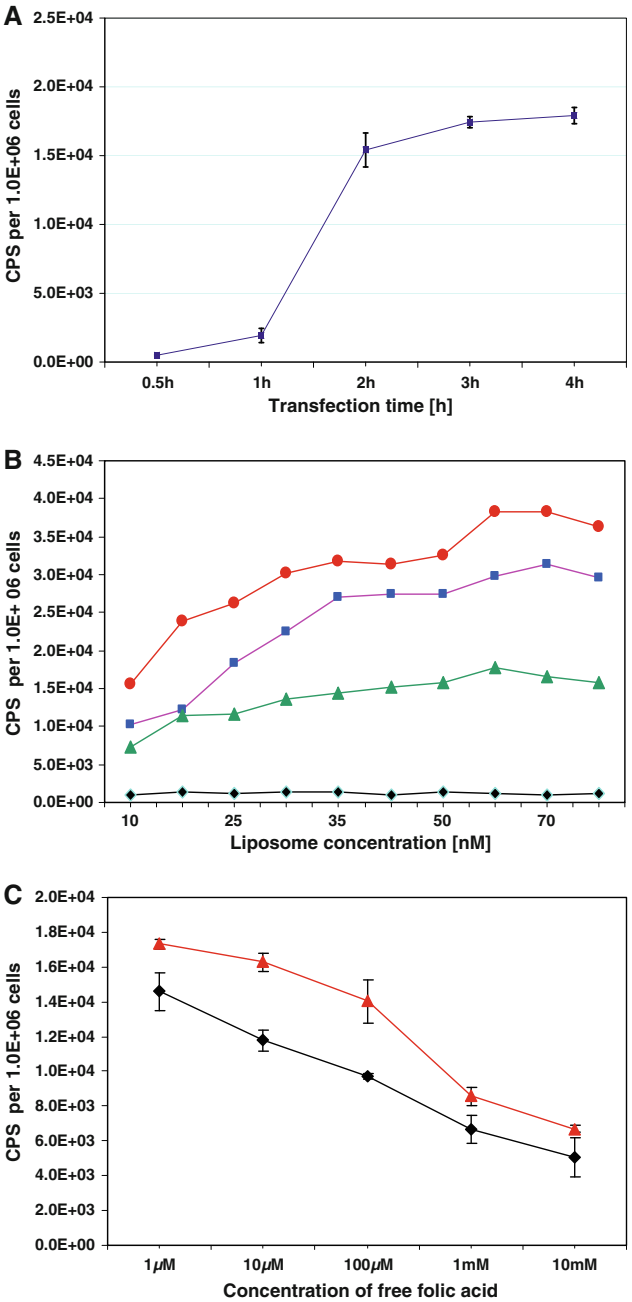

Fig. 2 Uptake of FTL liposomes (**a**), internalization of FTL liposomes as a function of temperature (**b**), uptake of FTL liposomes in the presence of folate (**c**). **a** FTL liposomes encapsulating calcein, a membrane-impermeant dye (10 mM internal concentration) were added to monolayers of KB cells and incubated at 37°C for various times. Cell monolayers were then lysed and cell-associated fluorescence measured. **b** KB cells were incubated with different concentrations of calcein-containing FTL liposomes for 4 h at two different temperatures. To determine the fraction of cell-associated liposomes that were internalized, the cells were stripped of their surface-bound liposomes. *Filled circles* cells treated at 37°C and washed with D-PBSA; *filled squares* cells treated at 4°C and washed with D-PBSA; *filled triangles* cells treated at 37°C and washed with saline (pH 3); *filled diamonds* cells treated at 4°C and washed with saline (pH 3), respectively. **c** The role of folate-binding receptors in the uptake of FTL liposomes was studied with KB and B16-F10 cell monolayers exposed to 60 nM liposomes in medium containing different concentrations of free folate. *Filled triangles* KB cells and *filled diamonds* B16-F10 cells. Diminution in liposome binding was then assayed as outlined above

was quantified. With incubation of the cells at 37°C, the acid wash removed liposomes only partially (Fig. 2b). The remaining liposomes were thus shielded against stripping with acidic saline. Incubation of KB cells at 4°C with FTL liposomes also resulted in the attachment of liposomes to the cells. These liposomes were, however, only surface attached and not internalized and could be eluted with the acidic saline wash (Fig. 2b). These results imply that the remaining fluorescence of the cells incubated at 37°C can be ascribed to internalized calcein-carrying liposomes, as binding to folate receptor occurs at both temperatures, whereas liposomes are endocytosed only at 37°C.

To shed light on the endocytosis of FTL liposomes by cells expressing folate receptors, the cell cultures (KB and B16-F10) were exposed to transfection medium containing FTL liposomes and increasing concentrations of folic acid. The uptake of the FTL liposomes was significantly reduced (by one order of magnitude) for both types of the studied cell lines (Fig. 2c), albeit not completely, which further suggests a no-folate-receptor component of endocytosis of these liposomes.

To check the effectiveness of the prepared liposome and peptide constructs in anticancer therapy, they were administered to mice bearing experimentally induced B16-F10 melanoma tumors. One treatment cycle with four intratumoral doses (250 μg each) of the previously optimized (not shown) therapeutic peptide and four intravenous doses of doxorubicin-carrying liposomes (equivalent to 40 μg of doxorubicin each) caused tumor growth to be inhibited compared with the control (Fig. 3a) and free doxorubicin alone (not shown). The chosen dose of doxorubicin resulted in minimal weight loss in the mice. The lifespan of the animals treated with this combined approach was extended compared with the control (Fig. 3b). We also tested liposomes with ligands for folate receptor, as malignant cells of epithelial origin are thought to express such receptors abundantly on their surface (e.g. Gabizon et al. 1999). One treatment cycle of combined therapy involving, besides the peptide, either FTL or PTL did not show, however, differences in tumor growth inhibition or animal survival (Fig. 3a, b).

Nonetheless, when the growth of the tumors was challenged by administering two treatment cycles of the vascular-destroying peptide and liposomal doxorubicin combination, the antitumor effects obtained against B16-F10 malignancy were different from those of the monotherapies or the single combined treatment cycle (Fig. 4a). No statistical differences were found between the

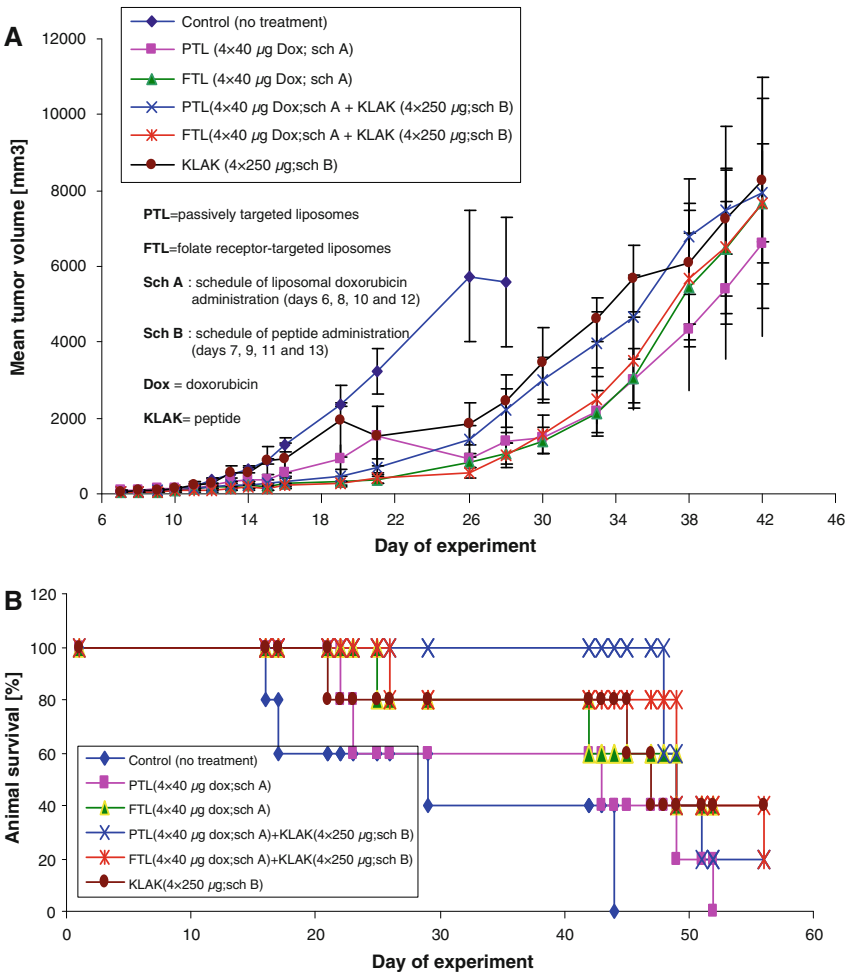

Fig. 3 **a** Inhibition of B16-F10 murine melanoma tumor growth following one cycle of therapy with doxorubicin-loaded liposomes and/or ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ antivascular peptide. C57BL/6 female mice were tumor inoculated with B16-F10 murine melanoma cells (for details see "Materials and Methods") and randomly tumor size-matched after 6 days ($n = 5$ mice per group). Monotherapies: mice receiving doxorubicin (40 μg)-loaded PTL (*filled squares*) or FTL (*filled triangles*) liposomes injected intravenously on days 6, 8, 10, and 12 post inoculation (160 μg doxorubicin total) or mice receiving 250 μg of antivascular peptide (*filled circles*) injected intratumorally on days 7, 9, 11, and 13 post inoculation (1 mg of peptide total). Combined therapies: mice receiving doxorubicin-loaded PTL (*multiplication symbol*) or FTL (*asterisks*) liposomes injected alternating with antivascular peptide (injection days as in the monotherapy schedules). *Control* mice receiving no therapeutic treatment (*filled diamonds*). *Other controls* free doxorubicin (4 × 40 μg) (toxic) and therapeutic agent vehicles (D-PBSA or no-doxorubicin PTL or FTL liposomes) are not shown. Data are mean values ± SD. Tumor growth curves were analyzed by the Mann–Whitney U test (significance level $p < 0.05$). **b** Kaplan–Meier survival analysis of B16-F10 melanoma tumor-bearing mice following one-cycle therapy with doxorubicin-loaded liposomes and/or ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ antivascular peptide. C57BL/6 female mice were inoculated with B16-F10 melanoma cells and treated with the therapeutic agents as described in **a** and in "Materials and Methods". Survival differences among various groups were analyzed using the log-rank test (significance level $p < 0.05$)

effectiveness of one and two treatment cycles involving only liposomes (either passively targeted or folate receptor-targeted). Two treatment cycles combining the FTL liposomes and the antivascular peptide caused a much stronger inhibition of tumor growth compared with the PTL and the peptide ($p = 0.0566$). Taken together, this might indicate that this effect is not simply the result of an increased cumulative dose of the drug-loaded liposomes. Animal survival was generally improved following two cycles of liposomal doxorubicin and peptide administrations compared with any single series (Fig. 4b). Especially worth noting is the statistically significant ($p = 0.0477$) difference in survival between the groups (single vs. repeated combination therapy) that received folate receptor-targeted liposomes. In the case of the groups (single vs. repeated combination therapy) that were treated with PTL liposomes, the difference was less clear ($p = 0.0573$). Further therapeutic experiments ought to clarify the contributions from various factors modifying the effects of such a combined strategy.

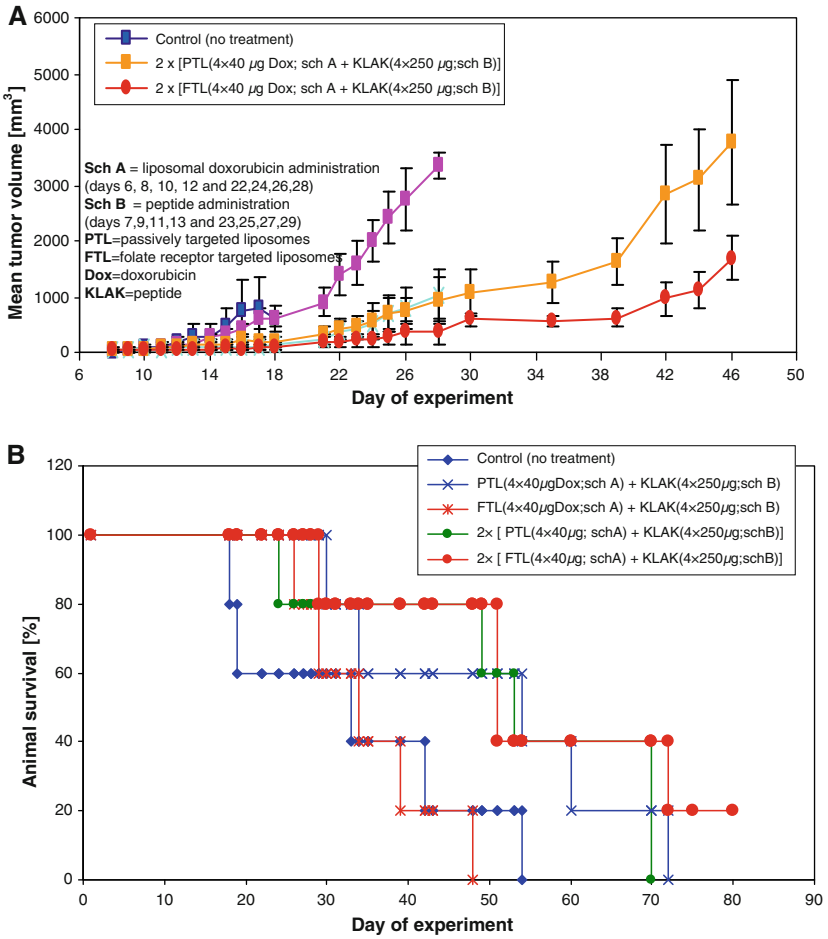

Fig. 4 **a** Inhibition of B16-F10 murine melanoma tumor growth following two cycles of therapy with doxorubicin-loaded liposomes and/or ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ antivascular peptide. C57BL/6 female mice were tumor inoculated with B16-F10 murine melanoma cells and randomly tumor size-matched as described in the legend of Fig. 3a. The first cycle of monotherapy with doxorubicin-loaded PTL or FTL liposomes or antivascular peptide as well as the first cycle of combined therapy involving doxorubicin-loaded PTL or FTL liposomes alternating with antivascular peptide were performed as described in Fig. 3a. Then, after a 10-day interval the same cycle was repeated for a second time. Totals of 320 μg of doxorubicin and 2 mg of peptide were thus delivered. For plot clarity, shown are only the results of two cycles of the combined liposomal doxorubicin + peptide therapies [PTL-based (*filled square*) and FTL-based (*filled circle*)]. *Controls* mice receiving no therapeutic treatment (*filled diamonds*). *Other controls* free doxorubicin (toxic), antivascular peptide, vehicles (D-PBSA or no-doxorubicin PTL or FTL liposomes) are not shown. Data are mean values ± SD. Tumor growth curves were analyzed by the Mann–Whitney *U* test (significance level $p < 0.05$). **b** Kaplan–Meier survival analysis of B16-F10 melanoma tumor bearing mice following two cycles of therapy with doxorubicin-loaded liposomes and/or ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ antivascular peptide. C57BL/6 female mice were inoculated with B16-F10 melanoma cells and treated with the therapeutic agents as described in **a** and in "Materials and Methods". Survival differences among the various groups were analyzed using the log-rank test (significance level $p < 0.05$). Extended survival of treated mice is seen following two cycles of combination therapy with the antivascular peptide and doxorubicin-loaded targeted liposomes compared with the other modes of administration. For details, see "Results and Discussion"

Finally, the H&E-stained sections prepared from tumors collected 24 h post single drug administration (peptide, liposomes, or both) were independently examined by two experienced pathologists and digital micrographs were analyzed with the help of an image analysis system. Differences were found in the type and extent of necrosis induced by the applied treatments. Peptide-mediated monotherapy resulted in a discernible multifocal cluster-like type of necrosis (19.8% of the section surface area, average single focal area ca. 3×10^5 μm^2), as seen in Fig. 5IA. Various stages of the necrotization process can be observed (Fig. 5IB). Liposomal doxorubicin monotherapy also produced multiple necrotic foci which were much smaller (average focal area ca. 0.94×10^5 μm^2) and diffused throughout the neoplastic tissue (ca. 15.5% of the tumor section area) as seen in Fig. 5IIA. Discohesion of tumor cells is seen next to already necrotized tissue (Fig. 5IIB). The difference in the distribution type of

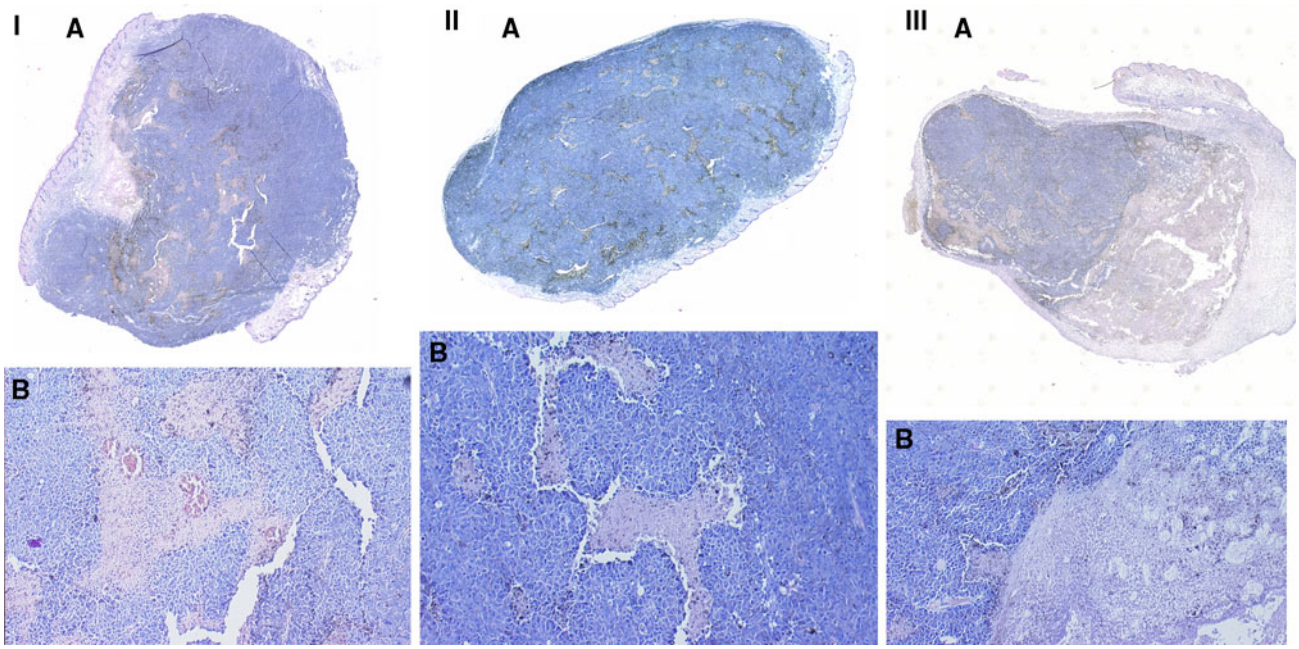

Fig. 5 Hematoxylin and eosin-stained paraffin sections of B16(F10) experimental tumors. C57BL/6 female mice were inoculated with B16-F10 melanoma cells as described in "Materials and Methods". The animals were killed and the tumors were excised 24 h after conclusion of a single therapeutic intervention with the studied therapeutic agent, performed essentially as described in the legend of Fig. 3a. The excised material was formalin-fixed and paraffin-embedded and the tumor sections were stained with H&E. The micrographs show whole tumor sections (**A**) and tumor section details (**B**); **I** following intratumoral administration of ACDCRGDCFC-GG-$_{D}$(KLAKLAK)$_2$ vascular-disruptive peptide, **II** following intravenous administration of doxorubicin-loaded PTL liposomes, **III** following administration of doxorubicin-loaded PTL liposomes and antivascular peptide. For comments, see "Results and Discussion"

necrotic foci (cluster vs. spread) following the peptide and liposomal treatments might in part reflect the different modes of administering the two drugs (intratumoral vs. intravenous). Combined therapy also resulted, as seen in Fig. 5IIIA, in an apparently diffused distribution of necrotic foci (average focal area ca. 1×10^5 μm^2), but, since it involved more than 57.6% of the tumor section area, it probably reflects the combined administration. Various stages and aspects of necrosis can be discerned in Fig. 5IIIB. The endothelial lining is seen to separate from the blood vessel walls, with blood clots visible in the vessels' lumen.

To sum up the results, pegylated liposomes carrying doxorubicin, both with and without folate ligands, seem to have mediated the transfer of this chemotherapeutic into the examined cancer cells in vitro although the FTL liposomes showed a greater extent of internalization in cells overexpressing folate receptors. Monotherapies of C57BL/6 mice bearing B16-F10 tumors with either antivascular peptide or liposomal doxorubicin caused some inhibition of tumor growth, although they did not result in an extension of the animals' lifespan. A single cycle of combined therapy with alternating administrations of liposomal doxorubicin and antivascular peptide led to tumor growth inhibition and slightly extended animal survival, with no advantage observed from the use of folate receptor-targeted liposomes. Two cycles of combined therapy brought several-fold stronger tumor growth inhibition (by one order of magnitude compared with the control) with a marked difference between combinations involving FTL versus PTL liposomes. The combined therapy approach also noticeably prolonged the survival of mice. The difference between one and two cycles of therapy was statistically significant for the antivascular peptide and folate receptor-targeted liposomes, whereas for the peptide and PTL liposomes it was not ($p = 0.0566$).

Intratumoral administration of a targeted antivascular peptide caused a rapid tumor vascular shutdown in the investigated tumor model due to the death of endothelial cells. The neoplastic cells were destroyed both directly through the $\alpha_V\beta_3$ receptor-mediated mechanism and indirectly through necrosis that followed the collapse of the vascular supply. Enhanced necrosis, seen especially after the combined liposomal doxorubicin–peptide treatment, further adds to the clogging of lymphatic drainage vessels. This should increase the therapeutic effect of tumor-entrapped liposomal doxorubicin. Cessation of treatment with the peptide led to rapid tumor relapse brought about by neoplastic cells that survived the first round of the combined treatment. The enhanced therapeutic effects seen

after two rounds of combined therapy appear to be linked to additional eradication of such surviving cancer cells. For the peptide treatment to be therapeutically effective, continuous administration would be required. Two rounds of the combined therapy were just a step in the right direction.

The curative potential of the vascular-destroying peptide in combination with pegylated folate receptor-targeted liposomes transferring doxorubicin seems promising. Further improvements might exploit the liposome clearance saturation phenomenon and ligand presence. As an example, in a recent study a doxorubicin dose escalation from 2.5 to 20 mg/kg resulted in a dose-dependent drug blockade of the reticuloendothelial system, saturation of liposomal doxorubicin clearance, prolonged circulation times, and a disproportionate increase in drug accumulation in murine tumors (Gabizon et al. 2002). In humans, while the pharmacokinetics of pegylated liposomal doxorubicin is not dose-dependent within the dose-range of 30–60 mg/m^2, there is evidence of a cycle-dependent effect that results in the inhibition of clearance when patients receive successive cycles of pegylated liposomal doxorubicin (Gabizon et al. 2008). Various improvements would bring less naïve injection schedules, optimizing the "window" for drug release in the tumor extracellular matrix and/or endocytosis of drug-loaded liposomes. Combinations of chemotherapy and a vascular-destroying strategy might be supplemented by yet other therapeutic approaches, for example anti-inflammatory. Since PTL have been known to localize in tumor-associated macrophages (TAMs) (Banciu et al. 2008), it might be possible to explore this phenomenon therapeutically by suppressing macrophage activity and inhibit TAM-mediated angiogenesis with clodronate-loaded liposomes. Finally, combined therapies might also make use of the ability of drug-loaded liposomes to penetrate drug-resistant cells to transfer suitable inhibitors targeting quiescent tumor stem cells, bypassing their MDR-transporter and detoxifying enzyme-mediated resistance.

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