Novel RGD lipopeptides for the targeting of liposomes to integrin-expressing endothelial and melanoma cells

Peter Hölig1, Miriam Bach1, Tina Völkel1, Thomas Nahde1, Sven Hoffmann1, Rolf Müller2 and Roland E. Kontermann1,3

1Vectron Therapeutics AG, Rudolf-Breitscheid-Strasse 24, 35037 Marburg, Germany and 2Institut für Molekularbiologie und Tumorforschung, Philippus-Universität, Emil-Mannkopff-Strasse 2, 35033 Marburg, Germany
3To whom correspondence should be addressed.
E-mail: kontermann@aol.com

RGD peptides targeting αv-integrins are promising ligands for the generation of vascular targeting agents. We isolated from phage display RGD motif libraries novel high-affinity cyclic RGD peptides by selection on either endothelial or melanoma cells. Although the starting sequences contained only two cysteine residues flanking the RGD motif, several of the isolated peptides possessed four cysteine residues. A high-affinity peptide (RGD10) constrained by only one disulfide bond was used to generate novel lipopeptides composed of a lipid anchor, a short flexible spacer and the peptide ligand conjugated to the spacer end. Incorporation of RGD10 lipopeptides into liposomes resulted in specific and efficient binding of the liposomes to integrin-expressing cells. In vivo experiments applying doxorubicin-loaded RGD10 liposomes in a C26 colon carcinoma mouse model demonstrated improved efficacy compared with free doxorubicin and untargeted liposomes.

Keywords: drug delivery/integrins/phage display/RGD peptides/vascular targeting

Introduction

Liposomes are carrier systems able to improve the pharmacological properties of a drug by altering pharmacokinetics and biodistribution, protecting the drug against degradation and reducing possible side effects (Drummond et al., 1999; Allen and Cullis, 2004). Selectivity and efficacy can be further improved through active targeting equipping the liposomes with ligands. For cancer therapy, ligands (e.g. antibodies, peptides or carbohydrates) recognizing tumor-associated antigens expressed on the surface of tumor cells have been employed (Forssen and Willis, 1998). However, in order to bind to cancer cells, liposomes have to extravasate from circulation and penetrate into the tumor tissue. Owing to a high interstitial pressure, extravasation is often inefficient (Mastrobattista et al., 1999).

Targeting of the tumor vasculature rather than the tumor cells themselves represents a promising new approach for cancer therapy (Matter, 2001). The applied vascular targeting agents are designed to deliver therapeutic substances specifically to the vasculature of tumors recognizing structures associated with tumor blood vessels (Halin et al., 2001; Thorpe et al., 2003). Targeting of the vasculature has several advantages over targeting of tumor cells (Augustin, 1998). There are no physiological barriers as endothelial cells are easily accessible for circulating carrier systems and penetration into the tumor is not necessary. Destruction of a few capillary endothelial cells affects a large number of tumor cells depending on them. Since all solid tumors are dependent on neovascularization in order to grow beyond a few millimeters in diameter, this approach should be broadly effective in treating tumors. Finally, endothelial cells are genetically stable and do not become resistant to the therapy (Boehm et al., 1997).

Various target structures suitable for vascular targeting have been described (Halin et al., 2001; Aina et al., 2002). Amongst these the αvβ3 and αvβ5 integrins have been extensively studied. These integrins are upregulated by proliferating endothelial cells of the tumor vasculature but are also found on different tumor cells including metastatic melanoma cells (Conforti et al., 1992; Gehlsen et al., 1992; Seftor et al., 1999; Varner and Cheresh, 1996). Targeting to these integrins can be achieved using RGD-containing peptides. A variety of linear and cyclic forms of these peptides have been investigated (Pasqualini et al., 1997; Wickham et al., 1997; Arap et al., 1998, Erbacher et al., 1999; DeNardo et al., 2000; Müller et al., 2001; Janssen et al., 2002; Schraa et al., 2002). One of the most potent RGD peptides (RGD4C; CDCRGDCFC) is composed of a central RGD motif structurally stabilized by two disulfide bonds (Koivunen et al., 1995). A recent study showed that this peptide can adapt two conformations depending on which cysteines form disulfide bridges, with the 1–4, 2–3 disulfide bond arrangement showing 10-fold stronger binding than the 1–3, 2–4 arrangement (Assa-Munt et al., 2001). Hence, in order to obtain correctly folded high-affinity RGD4C, a two-step oxidation process is required.

The phage display technology represents a powerful tool to evolve sequences with improved affinity and specificity (Szardenings, 2003). Here, we isolated novel high-affinity RGD peptides applying phage display RGD motif libraries selected on endothelial or melanoma cells. These selections resulted in several high-affinity RGD peptides constrained by only a single disulfide bond. One of these peptides with a similar affinity as RGD4C was used to produce novel lipopeptides allowing direct incorporation into the lipid bilayer of liposomes via the lipid anchor. These RGD liposomes showed efficient and specific binding to integrin-expressing cells. Furthermore, we could demonstrate that these RGD liposomes led to improved anti-tumor effects in a subcutaneous tumor model.

Materials and methods

Materials

All lipids were purchased from Avanti (USA). Peptides were purchased from Jerini (Berlin, Germany) and lipopeptides were synthesized by emc microcollections (Tübingen, Germany). Cysteine-containing RGD peptides were produced in oxidized form. RGD4C peptide containing four cysteine residues was synthesized by emc microcollections (Tubingen, Germany).
oxidized in the 1–4, 2–3 configuration as described (Assa-Munt et al., 2001). HUVECs and HDMECs were obtained from Promocell (Germany).

**Construction of RGD motif libraries**

Two RGD motif libraries displaying the randomized peptides as g3p fusion protein were generated: CPL4b (D G X X X C R G D C X X X . . . ) and CPL4c (X X C R G D C X X X . . . ), with the X residues (=L, V, F, Y, W, H, D, E, C, Q, S, G, A or P) encoded by the triplet BKN. Oligonucleotides CPL4bFor (5’-AGT TTC TGC GGC CGC CCC TCC MNV MNV ACA GTC GCC AGC MNU MNV MNV CCC GTC TGC CAT GGC CGG CTG GGC CGC ATA GAA AGG-3’) and CPL4cFor (5’-AGT TTC TGC GCC GCG TCC TCC MNV MNV ACA GTC GCC AGG CAT GCA MNV MNV MNV TGC CAT GGC CGG CTG GGC CGC ATA GAA AGG-3’) encoding the randomized RGD peptide fragment were converted into double-stranded DNA using the Klenow fragment of *Escherichia coli* DNA polymerase and primer SfIBack2 (5’-CCT TTC TAT GCC GCC CAG CCG GCC ATG-3’) for annealing. Template oligonucleotides (100 pmol) were mixed with 300 pmol of primer, annealed by heating for 5 min at 94°C and slowly cooled to room temperature. Five units Klenow fragment and dNTPs to a final concentration of 250 μM were then added and incubated for 15 min at 37°C. Reaction was stopped by incubation for 10 min at 65°C. DNA was digested with SfiI and NotI and ligated into phage vector fdVT3. Ligation products were transformed into electrocompetent TG1 (Stratagene) and plated on to 2 × TY, tet plates. Library sizes were 1 × 10⁸ for CPL4b and 4 × 10⁹ for CPL4c. Phages were produced by growing bacteria overnight at 30°C and precipitation of phage from culture supernatant with 20% PEG-6000–2.5 M NaCl.

**Cell selections**

Phages were pre-absorbed either on ~10⁶ HEK293 (selection on human cells) or NIH 3T3 (selection on murine cells) in PBS, 2% skimmed milk powder (2% MPBS) for 1 h on ice. Cells were pelleted and supernatant was added to 10⁶ target cells in 2% MPBS. After incubation for 1 h on ice, cells were washed three times with 2% MPBS and three times with PBS and bound phage were eluted by incubation of the cell pellet with 1 ml of 100 mM triethylamine for 8 min. The solution was neutralized with 0.5 ml of 1 M Tris–HCl pH 7.4, centrifuged for 2 min and the supernatant added to 10 ml of log-phase TG1 (OD₆⁰₀ = 0.4–0.5) grown at 37°C. After incubation for 60 min at 37°C, cells were plated on to 2 × TY, tet plates and incubated overnight at 30°C. Phage were recovered as described above.

**Preparation of liposomes**

All lipids were dissolved in CHCl₃ and lipopeptide was dissolved in CHCl₃–MeOH (1:1). Lipids (cholesterol, sphingomyelin, phosphatidylethanolamine and phosphatidylcholine at molar ratios of 3.5:1.8:1.5:3.2) and lipopeptide were mixed at the indicated molar ratios and the solvents were removed by rotary evaporation at 34°C for 30 min and drying under vacuum for at least 1 h. The lipid film was hydrated with PBS for FACS analysis or with 300 mM citrate buffer pH 4 for remote loading with doxorubicin. The final concentration was 10 μmol lipid/ml buffer (for FACS and *in vivo* experiments) or 50 μmol lipid/ml buffer (for loading experiments). The hydration was performed in a water-bath at 34°C for 30 min. The resulting unilamellar vesicles were extruded (LiposoFast Extruder) 21 times through a polycarbonate filter (Armatis) with a pore size of 50 nm. The size of the large unilamellar vesicles was measured by dynamic light scattering using a Malvern Autosizer.

**Fluorescence-activated cell sorting (FACS) analysis of targeted liposomes**

Liposomes were prepared as described above incorporating 0.3 mol% rhodamine-labeled phosphatidylethanolamine (Rh-DPPE) into the lipid bilayer. Cells were detached from cell culture dishes using 0.02% EDTA (Sigma). About 150 000–200 000 cells were incubated with liposomes (50 nmol lipid) in the presence of culture medium for 1 h. After washing with PBS, 1% BSA cells were resuspended in 400 μl of PBS and analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

**Competition experiments**

HUVECs were detached from cell culture plate with EDTA and resuspended in EGM-2 medium. About 10⁵ cells were incubated with RGD phages (~10¹¹ t.u.) or 50–100 nmol rhodamine-labeled liposomes (see below) and the indicated concentration of free peptide/competitor for 1 h at 4°C. Cells were washed in ice-cold PBS and bound phages were detected with HRP-conjugated anti-M13 antibody (Pharmacia) and PE-labeled secondary antibody. Binding was then analyzed by FACS. For titration experiments, bound phages were detected with HRP-conjugated anti-M13 antibody and subsequent incubation with TMB–H₂O₂ as substrate as described (Kontermann, 2001).

**Internalization studies**

Cells grown on cover-slips were washed twice with PBS and incubated with rhodamine–DPPE-labeled liposomes (50 nmol lipid) in the presence of culture medium for 2 h at either 4 or 37°C. After washing with cold PBS, cells were fixed using 3.7% formaldehyde in PBS and mounted with Mowiol. Staining was analyzed by fluorescence microscopy. For inhibition studies with free peptide, RGD10 peptide at a concentration of 100 μM was added to the medium. For co-localization studies we used rhodamine–DPPE-labeled RGD10–LP3 liposomes (1 mol% lipopeptide) containing carboxyfluorescein (CF), CF (Sigma-Aldrich, Deisenhofen, Germany) was encapsulated into liposomes by adding 10 mM CF to the hydration buffer. Unencapsulated CF was removed by gel filtration on a Sepharose 4B column.

**Remote loading of doxorubicin into liposomes**

The liposomes were prepared as described above. In order to load doxorubicin into the liposomes, a transmembrane pH gradient was generated. The first 200 μl of the liposome suspension (50 μmol lipid/ml) was diluted with 200 μl of phosphate buffer (pH 7.4, 10 mM). To achieve the pH gradient (intraliposomal media, pH 4; extraliposomal media, pH 7.5), NaOH (2 M) was added to the extraliposomal media. This suspension was heated in a water-bath at 60°C for 5 min. Then the doxorubicin–HCl solution was added (molar ratio doxorubicin to lipids = 0.2:1) and the volume was diluted to 1 ml with phosphate buffer to obtain a final lipid concentration of 10 μmol/ml. The loading process was allowed to proceed for 15 min at 60°C and 15 min at room temperature. To remove unencapsulated...
doxorubicin, the liposomal suspension was applied to VivaSpins (2 mL, VivaScience) and centrifuged for 20 min at 2400 r.p.m. (1200 g). The encapsulated doxorubicin content was measured with a spectrophotometer at 490 nm.

**Pharmacokinetics**

Liposomes labeled with [3H]cholesterol oleyl ether ([3H]COE) were injected into the tail vein of nude mice and blood samples were taken at the indicated time points. After 6 h the mice were killed and the radioactivity in various organs was measured. For calculations of blood clearance, the 3 min value was taken as 100% of the injected dose. The initial half-life \((t_{1/2})\) was calculated using the values between 3 and 20 min and the terminal half-life \((t_{1/2})\) was calculated using the values between 60 and 360 min. For further comparison we also calculated the 50% values, i.e. the time after which 50% of the injected dose was eliminated from the bloodstream.

**In vivo tumor therapy**

Nude mice (five animals per group) were injected i.d. with \(5 \times 10^5\) C26 murine colon carcinoma cells. Tumor-bearing mice were treated with free or liposome-encapsulated doxorubicin at a concentration of 4 mg/kg body weight. Mice received three injections i.v. at days 0, 2 and 5. Tumor volumes were monitored and calculated using the formula \(ab^2/2\) (\(a = \text{tumor length}, b = \text{tumor width}\)). Two independent sets of experiments were performed.

**Results**

**Selection of novel RGD peptides**

Two composed peptide libraries (CPL4b, CPL4c) containing an RGD motif flanked by cysteine residues were generated in a multivalent phage display format. Six amino acid positions, three preceding and three following the central –CRGDC– motif were randomized introducing the codon BNK (B = G, T, C; K = G, T; N = A, C, G, T) encoding all amino acids except Lys, Ile, Met, Thr and Asn. Lysine residues were avoided in order to facilitate a possible site-specific chemical coupling through the N-terminal amino group. CPL4b differed from CPL4c by an Asp–Gly sequence at the C-terminus. Library sizes were \(1 \times 10^9\) for CPL4b and \(4 \times 10^8\) for CPL4c with 83% positive clones for both libraries.

Phages were selected on human dermal microvascular endothelial cells (HDMEC), a human melanoma cell line (MeWo) or a mixture of murine melanoma cell lines B16.F1 and B16.F10. Four to three rounds of selections were performed for each cell line. Fluorescence-activated cell sorting (FACS) analysis of polyclonal phage preparations showed enrichment of cell-binding phage after 2–3 rounds. Interestingly, phage selected against HDMEC also reacted with MeWo. In addition, cross-reactivity was observed for phage selected on MeWo with B16.F1/B16.F10. Phage selected on B16.F1/B16.F10 showed some weak binding to MeWo. No binding was observed with a negative control cell line (HEK293).

By screening single clones of round 3 or 4, we identified 15 different sequences, with some sequences occurring several times (Table I). Except for two peptides selected on MeWo, all peptides were from the CPL4b library. The identified sequences can be divided into two groups. Peptides of group one (4C-RGD peptides) contain four cysteine residues either at positions 4, 6, 10 and 12 (peptides 1–5) or at positions 5, 6, 10 and 12 (peptide 6), with positions 6 and 10 corresponding to the fixed cysteine residues. Hence these peptides resemble the structure of peptide RGD4C with the sequence ACDCRGNDCFCG (Koivunen et al., 1995). Peptides 1–5 all contain a hydrophilic residue at position 5 (Q, E, H) and a hydrophobic residue at position 11. (L, F). Peptides of group two (2C-RGD peptides) contain only the two fixed cysteine residues at position 6 and 10. Interestingly, four out six peptides have an arginine residue at position 4 and all have an aspartate or glutamate residue at position 12 (peptides 7–12). These peptides prefer an alanine residue at position 3 and a valine residue at position 11.

**Characterization of peptide RGD10**

Analysis of 4C and 2C peptides for binding to endothelial or melanoma cells showed binding activities similar to or even better than phage-displayed RGD4C, which was included as control. For further analysis we selected 2C-RGD peptide RGD10 (DGARYCAGDGFDCG). Phages displaying RGD10 bound with strength comparable to RGD4C phages to HDMEC. However, RGD10 contains only two cysteine residues, which facilitates synthesis of oxidized peptides. Furthermore, this peptide contains two aromatic residues allowing detection at 280 nm. An RGD10 peptide was synthesized with the sequence GARYCRGDCFDG, thus lacking the vector encoded C-terminal aspartate residue and resulting in a neutral net charge. Competition experiments with free RGD10, RGD4C and a control peptide with the sequence SIYGPL (SIG peptide) described to bind to endothelial cells (Nicklin et al., 2000) showed that both RGD peptides competed for binding of RGD10 or RGD4C phage to HUVECs whereas the SIG peptide did not cause any inhibition of phage binding (Figure 1). Thus, RGD10 binds to the same epitope as RGD4C known to be a high-affinity ligand of \(\alpha_v\beta_3\) integrin. In addition, this study demonstrates that the SIG peptide is not a ligand for RGD-binding integrins. A titration for competition of free

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**Table I. Isolated RGD peptides**

<table>
<thead>
<tr>
<th>No.</th>
<th>Target cells</th>
<th>Sequence</th>
<th>Round</th>
<th>Frequency</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>HDMEC, MeWo</td>
<td>DGV C Q CRG DC FC P ...</td>
<td>3, 4</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>HDMEC</td>
<td>DGV C Q CRG DC LC V ...</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>HDMEC</td>
<td>DGP C H CRG DC LC Q ...</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>HDMEC</td>
<td>DGP C E CRG DC LF P ...</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>MeWo</td>
<td>DGE C Q CRG DC LC P ...</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>B16F1/F10</td>
<td>DGE AC CRG DC VCE ...</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>RGD4C(1)*</td>
<td>ACD CRG DC FC G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGD4C(2)*</td>
<td>ACD CRG DC LC G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C-RGD peptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>B16F1/F10</td>
<td>DGS RL CRG DC VDA ...</td>
<td>3</td>
<td>1</td>
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<td>DGAR V CRG DC V DQ ...</td>
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<td>2</td>
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<tr>
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<td>1</td>
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<td>3</td>
<td>1</td>
</tr>
<tr>
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<td>3</td>
<td>4</td>
</tr>
<tr>
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</tr>
<tr>
<td>13</td>
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</tr>
<tr>
<td>14</td>
<td>MeWo</td>
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<td>3</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>MeWo</td>
<td>DGP FG CRG DC S Q E ...</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

*Peptide selected from a random phage display peptide library against \(\alpha_v\beta_3\) integrin (Koivunen et al., 1995).
peptide with binding of RGD10 phage to HDMEC gave identical IC₅₀ values of ~0.2 μM for RGD10 and RGD4C.

**Binding of RGD10-lipopeptide containing liposomes to endothelial and melanoma cells**

We designed a novel lipopeptide structure composed of a 1,2-dipalmitoyl-glycero-3-succinyllysine anchor to which the RGD10 peptide was coupled using one or three copies of 8-amino-3,6-dioxaoctanoic acid (doo) as spacer (Figure 2). With liposomes containing these RGD10 lipopeptides (RGD10-LP1 with a 1-doo spacer, RGD10-LP3 with a 3-doo spacer) we could detect specific binding to endothelial cells as well as melanoma cells. Strong binding was observed for human umbilical vein endothelial cells (HUVEC) and to a weaker extent for murine melanoma cell line B16.F10 (Figure 3A). Analysis of the mean fluorescence revealed a 2- to 3-fold stronger binding to HUVECs compared with B16.F10. Binding experiments with liposomes containing 0.1, 1 or 5 mol% RGD10-LP1 or RGD10-LP3 showed that binding to these cells increased with increasing concentration of lipopeptides (Figure 3A). A 1.5- to 2-fold stronger binding was observed for 1 mol% LP1 or LP3 compared with 0.1 mol% lipopeptide. In addition, liposomes containing RGD10-LP3 showed increased binding (1.5- to 2-fold) to HUVEC or B16.F10 compared with liposomes containing RGD10-LP1, indicating that the longer doo spacer improves binding to the cell surface. All lipopeptide-containing liposomes had an average size of 76 ± 2 nm. Thus the incorporation of 1 mol% lipopeptide corresponds
to a theoretical value of ~300 surface-displayed ligands per liposome.

Weak binding of RGD10-LP3 liposomes containing 1 mol% lipopeptide was also seen with MeWo cells (Figure 3B). In contrast, no improved binding compared with plain liposomes was observed with A431 cells included as negative control (Figure 3B).

In order to demonstrate further that binding is mediated by the RGD10 peptide, we performed various competition experiments. Synthetic peptides RGD10 or RGD4C, but not SIG included as an irrelevant control peptide, completely inhibited binding of RGD10-LP1-containing liposomes to HUVEC or B16.F10 (Figure 4A–C). In addition, no inhibition of binding was observed with a RGE peptide, further demonstrating specificity of the RGD10 ligand. Incubation of RGD10-liposomes with an excess amount of a molecule consisting of five doo units (5-doo) did not inhibit binding to HUVECs, indicating that the spacer region of the lipopeptide consisting of one or three doo units does not contribute to cell binding (Figure 4D).

In a further experiment, we compared binding of RGD10-LP3 liposomes to HUVECs with binding of liposomes containing 1 mol% of a mutated lipopeptide (RGE-liposomes). In this lipopeptide the aspartate of the RGD motif was substituted by a glutamate residue resulting in an inactive RGE sequence (Cherny et al., 1993). This mutation completely abolished binding of lipopeptide-containing liposomes to HUVECs (Figure 4E). Both liposomal formulations did not bind to C26 tumor cell line included as a further negative control (Figure 4F).

Fluorescence microscopy of HUVECs incubated with RGD10-LP3 liposomes at 37°C for 1 h revealed a perinuclear staining pattern indicative for internalized liposomes. In contrast, only cell surface staining was observed after incubation at 4°C (Figure 5A). In these experiments, no staining was observed with liposomes lacking the RGD10 lipopeptide.
Internalization of RGD10-LP3 liposomes at 37°C could be completely inhibited by coinubcation with 100 μM free RGD10 peptide, indicating that internalization requires binding to integrins (Figure 5B). Further studies with RGD10-LP3 liposomes containing encapsulated carboxyfluorescein (CF) demonstrated that liposomes are internalized in intact form as indicated by co-localization of red fluorescent lipid marker and green fluorescent content (Figure 5C).

**Pharmacokinetics of RGD10-LP3 liposomes**

Pharmacokinetics were analyzed in nude mice injected with [3H]COE-labeled liposomes containing different amounts of...
lipopeptide (Figure 6). Liposomes containing 1 mol% RGD10-LP1 or RGD10-LP3 were rapidly eliminated from the bloodstream with a $t_{1/2}$ of $\sim$9–10 min. Reduction of the amount of lipopeptide incorporated into liposomes to 0.1 mol% prolonged serum residence time to 102 min as calculated for the 50% value ($t_{1/2}=44$ min, $t_{1/2}=9.2$ h). This value is close to the serum residence time of liposomes lacking lipopeptides, which is $\sim 108$ min. Measuring organ distribution 6 h post-injection we found for all lipopeptide-containing formulations strong accumulation in the liver (51–54% of the injected dose) and spleen (4–11% the injected dose) and only marginal accumulation in the kidneys and lung (0.1–0.4% of the injected dose). In contrast, untargeted liposomes showed reduced accumulation in the liver (13%) and spleen (3%).

One method to prolong the serum residence time of liposomes is the incorporation of PEG into the liposome envelope. For this purpose we analyzed a liposomal formulation containing 1 mol% RGD10-LP3 and 5 mol% DPPE-PEG2000 incorporated into the lipid bilayer. Binding studies showed, however, that the PEG chains completely inhibited RGD10-mediated binding of the liposomes to cells (not shown).

**Anti-tumor effects of doxorubicin-loaded RGD10 liposomes**

Based on the pharmacokinetic data, we used RGD10-LP3 liposomes containing 0.1% lipopeptide for analysis of in vivo anti-tumor effects. For this purpose, liposomes were loaded with doxorubicin and analyzed in a C26 colon carcinoma mouse model. These experiments revealed an improved anti-tumor effect of doxorubicin-loaded RGD10-LP3 liposomes compared with free doxorubicin and to untargeted liposomes (Figure 7). After 9 days of treatment with targeted liposomes, tumor growth was inhibited by 52% and at day 11 by 31% compared with mice treated with untargeted liposomes (calculated from the tumor volumes derived from two experiments). However, these differences were found to be not statistically significant ($p=0.13$ and 0.28 for days 9 and 11, respectively). The improved anti-tumor effect was also reflected by a prolonged mean survival time, which was 18 days for animals treated with doxorubicin-loaded RGD10-LP3 liposomes, compared with 15 days for untargeted liposomes.

**Discussion**

RGD peptides are widely used ligands for the targeting of integrin-expressing cells (Kolonin et al., 2001). We could isolate a panel of novel RGD peptides by selection of a motif library on endothelial or melanoma cells. The application of RGD motif libraries with a fixed CRGDC motif directs the phage towards RGD-binding receptors facilitating the enrichment of peptides with optimized amino acid residue surrounding the central RGD motif.

Ten out of 23 analyzed peptides contained four cysteines, with a total of six different sequences. These peptides resemble the structure of peptide RGD4C known to exhibit high-affinity $\alpha_v\beta_3$-integrin binding, with the four cysteines at identical positions (except peptide 6) and hydrophilic amino acids at positions 5 and hydrophobic residues at position 11, both positions adjacent to the two central cysteine residues. RGD4C was selected by phage display on purified $\alpha_v\beta_3$-integrin from a constrained CX-C peptide library (Koivunen et al., 1995). Thus this library had, in contrast to our RGD motif library, the outer cysteine residues at fixed positions. Structural data for RGD4C revealed that high-affinity binding requires the formation of disulfide bonds between cysteines 1 and 4 and between cysteines 2 and 3. This study also showed that phenylalanine forms a hydrophobic pocket possibly involved in interactions with a hydrophobic patch within the receptor (Assa-Munt et al., 2001). As observed in our selections, a hydrophobic residue flanking the RGD motif at the carboxyl side was found to be dominant in phage display selections (Koivunen et al., 1995).

Interestingly, we also isolated a panel of 2C-peptides selected either on HDMEC or the murine melanoma cells. These peptides showed a strong overall similarity (consensus sequence: A R Y/L C R G D C V D). All peptides contained a negatively charged residue (Asp, Glu) at position +3 from the RGD motif and four out of six peptides contained a positively charged residue (Arg) at position +3. In the 4C-peptides these positions are occupied by cysteine residues. One can speculate that these charged residues either stabilize a favored peptide structure by ionic interactions or are directly involved in receptor binding. In this context, it is noteworthy that several RGD-containing proteins, e.g. disintegrins such as echistatin,
eristostain or decorsin, with specificity for integrins consisting of $\alpha_v$ and/or $\beta_3$ exhibit a similar composition (Wierzbicka-Patynowski et al., 1999). These molecules possess a positively charged amino acid (mainly arginine) at position $-3$ from the RGD motif (as observed in our 2C peptides) and one or more aspartate residues C-terminal (positions $+1$ to $+4$) of the RGD motif (see Table II). In addition, a selection of integrin-binding peptides from a constrained X$_3$RGDX$_4$ library was recently described (Li et al., 2003). In this study all except one peptide selected on $\alpha_v\beta_3$-integrin possessed an aspartate residue at position $+2$ and 60% of the peptides had an arginine residue at position $-3$. These peptides showed a preferred binding to $\alpha_v\beta_3$-integrin. In contrast, peptides selected on $\alpha_{IIb}\beta_3$ lacked negatively charged residues C-terminal of the RGD motif but all contained an arginine residue at position $-2$ or $-3$. Although these peptides are not constrained by cysteine residues directly adjacent to the RGD motif, these findings indicate that a positively charged residue N-terminal and a negatively charged residue C-terminal of the RGD motif may favor binding to $\alpha_v\beta_3$-integrin. This is further supported by data from selection of a library of the tenth fibronectin type III domain that contained a randomized RGD loop (XRGDX$_4$) (Richards et al., 2003). A RGDWXE consensus sequence was identified in these experiments conferring enhanced affinity and specificity for $\alpha_v\beta_3$-integrin. Mutational analysis indicated that the tryptophan residue might be important in conferring specificity to $\alpha_v$, whereas the glutamic acid residue may confer specificity to $\beta_3$.

Although we have not analyzed the specificity of the selected peptides for the various integrins, they have a clear specificity for cells known to express high levels of $\alpha_v$-integrins. Experiments with 2C peptide RGD10 demonstrated a similar affinity for endothelial cells as RGD4C. This finding and the fact that peptides structurally similar to RGD4C were selected underline the power of the motif library approach in finding high-affinity peptides. Compared with RGD4C, synthesis of RGD10 is facilitated by the presence of only two cysteines, which makes an elaborate two-step oxidation using two different protection groups unnecessary (Assa-Munt et al., 2001).

We could not identify a cell-type specific pattern of the 4C-peptides or 2C-peptides selected on endothelial or melanoma cells and one peptide (RGD1) was even found in selections on HDMEC and MeWo. However, three peptides (RGD13, -14 and -15) selected on the human melanoma cell line MeWo had a sequence surrounding the central RGD motif different from the other peptides. Two of these peptides were selected from library CPL4c lacking the N-terminal DG sequence. It might be possible that these peptides recognize integrins different from those expressed by HDMECs or B16.F10 cells. Different integrins have been described to be upregulated in melanoma cells and to correlate with melanoma cancer progression (Kramer et al., 1991; Seftor et al., 1999; Kageshita et al., 2000). Further studies are needed to demonstrate the specificity of the peptides for certain integrins.

Using lipopeptides containing the RGD10 peptide, we could transfer ligand specificity on to liposomes. These liposomes showed the same binding activity as observed for the phage-displayed or free peptide. The lipopeptides used in this study contain a flexible and hydrophilic spacer consisting of one or three copies of 8-amino-3,6-dioxoaoctanoic acid (2-[2-(amino)ethoxy]ethyoxicetic acid). Although we did not compare binding with lipopeptides lacking the diso spacer moiety, our experiments indicate that the spacer length is critical for efficient binding. This is in accordance with findings for liposomes containing glycolipids consisting of $\beta$-galactoside and ethylene glycol-based spacers. In these experiments a spacer length of 13 or 22 atoms resulted in optimal agglutination of the liposomes with ricin, whereas shorter spacers of four or seven atoms did not lead to agglutination or only at very high phospholipid concentrations (Slama and Rando, 1980). The 1-doo and 3-doo spacers used in our study, including the succinilysine moiety of the lipid anchor, can span a distance of $\sim 2.5$ and $\sim 5$ nm, respectively. This puts the ligands away from the lipid head groups extruding $\sim 0.5$ nm into the aqueous phase.

Recently, another study described the generation of integrin-targeting RGD liposomes for chemotherapeutic applications. These liposomes contained a thiolated constrained RGD peptide coupled to maleimide-PEG liposomes (Jannsen et al., 2003; Schifferlers et al., 2003). As observed in our study, these RGD liposomes showed specific binding to endothelial cells and improved anti-tumor effects testing encapsulated doxorubicin in a C26 tumor model.

Pharmacokinetics were strongly affected by the presence of lipopeptides. The incorporation of 1 mol% lipopeptide reduced the serum half-life (50% value) to a few minutes. Although we did not analyze conjugates lacking the peptide ligand, it is most likely that this reduction is caused by the peptide ligand. Nevertheless, the reduction of lipopeptide concentration to 0.1 mol% led to a residence time almost identical with that of unconjugated liposomes, although an increased accumulation in various organs such as liver and spleen was observed. Similar observations have also been described for RGD–PEG liposomes (Schifferlers et al., 2003), indicating that this increased accumulation is mediated by the RGD peptides.

In vivo therapy studies demonstrated that the RGD liposomes caused a reduced tumor growth compared with unconjugated liposomes. This finding indicates that RGD liposomes targeting integrins on endothelial cells are efficacious carrier systems for the delivery of drugs to the tumor vasculature. Compared with chemical coupling of peptide ligands to a liposome surface, the usage of lipopeptides allows for the

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**Table II.** Comparison of selected 2C RGD peptides with other RGD sequences from various proteins with known binding activity for $\alpha_v$ and/or $\beta_3$ integrins: positively and negatively charged amino acid residues flanking the central RGD motif are highlighted with gray boxes.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGD11</td>
<td>DGALYCRGDCVDQ</td>
</tr>
<tr>
<td>RGD12</td>
<td>DGAFLCRGDCVDQ</td>
</tr>
<tr>
<td>RGD7</td>
<td>DGMLRCRGDCVA</td>
</tr>
<tr>
<td>RGD9</td>
<td>DGRSCKELCVA</td>
</tr>
<tr>
<td>RGD10</td>
<td>DGAYCERGDCFDG</td>
</tr>
<tr>
<td>RGD8</td>
<td>DAGVRGDCVDQ</td>
</tr>
<tr>
<td>Echistatin</td>
<td>CKRARGLDMDYHC</td>
</tr>
<tr>
<td>Eristostatin</td>
<td>CNVARCDNDDYHC</td>
</tr>
<tr>
<td>Albolabrin</td>
<td>CNPARGDLDHC</td>
</tr>
<tr>
<td>Decorin</td>
<td>CNFPRGAFPYC</td>
</tr>
<tr>
<td>Kistin</td>
<td>CNPREDMPDRC</td>
</tr>
<tr>
<td>Triflavin</td>
<td>CNTRARGFPDRC</td>
</tr>
<tr>
<td>Li et al. (2003)$^a$</td>
<td>RVPGRGSDLT</td>
</tr>
<tr>
<td>Li et al. (2003)$^b$</td>
<td>RSGRDSHR</td>
</tr>
<tr>
<td>FNfn10-3CLH$^b$</td>
<td>PRGDNMEG</td>
</tr>
</tbody>
</table>

$^a$Peptide displayed in the context of tendamistat selected by phage display against $\alpha_v\beta_3$ integrin (Li et al., 2003).

$^b$RGD loop of an engineered fibronectin type III domain selected by phage display against $\alpha_v\beta_3$ integrin (Richards et al., 2003).
production of targeted liposomes with defined properties. Hence the lipopeptides described in this study should be advantageous for the development of clinically useful carrier systems.

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References


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