

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/6427928>

Anginex-Conjugated Liposomes for Targeting of Angiogenic Endothelial Cells

ARTICLE in BIOCONJUGATE CHEMISTRY · MAY 2007

Impact Factor: 4.51 · DOI: 10.1021/bc060316h · Source: PubMed

CITATIONS

36

READS

43

6 AUTHORS, INCLUDING:



Ricardo J M G E Brandwijk

Maastricht University

12 PUBLICATIONS 1,001 CITATIONS

SEE PROFILE



Klaas Nicolay

Technische Universiteit Eindhoven

495 PUBLICATIONS 15,280 CITATIONS

SEE PROFILE



Victor L Thijssen

VU University Medical Center

76 PUBLICATIONS 2,049 CITATIONS

SEE PROFILE

Anginex-Conjugated Liposomes for Targeting of Angiogenic Endothelial Cells

Ricardo J. M. G. E. Brandwijk,[#] Willem J. M. Mulder,[§] Klaas Nicolay,[§] Kevin H. Mayo,[†]
Victor L. J. L. Thijssen,[#] and Arjan W. Griffioen^{*,*}

Angiogenesis Laboratory, Research Institute Growth and Development (GROW), Department of Pathology, Maastricht University & University Hospital, Maastricht, The Netherlands; Biomedical NMR, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands, and Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455. Received October 9, 2006;
Revised Manuscript Received February 1, 2007

Identification of a tumor angiogenesis specific ligand would allow targeting of tumor vasculature. Lipidic vehicles can be used to deliver therapeutic agents for treatment of disease or contrast agents for molecular imaging. A targeting ligand would allow specific delivery of such formulations to angiogenic sites, thereby reducing side effects and gaining efficiency. Anginex, a synthetic 33-mer angiostatic peptide, has been described to home angiogenically activated endothelium, suggesting an ideal candidate as targeting ligand. To investigate this application of anginex, fluorescently labeled paramagnetic liposomes were conjugated with anginex. Using phase contrast and fluorescence microscopy as well as magnetic resonance imaging (MRI), we demonstrate that anginex-conjugated liposomes bind specifically to activated endothelial cells, suggesting application as an angiogenesis targeting agent for molecular targeting and molecular imaging of angiogenesis-dependent disease.

INTRODUCTION

Lipid-based vesicles, such as liposomes, can be used as therapeutic vehicles for transport, e.g., drugs, DNA, proteins/peptides, and antibodies (1, 2), or for diagnostic purposes such as the delivery of MRI contrast agents (3). However, liposomes have a relatively short circulation time because they are taken up by the mononuclear phagocyte system (MPS) (4–6). In order to increase their circulation time, hydrophilic carbohydrates or polymers (e.g., PEG) can be included in the lipidic formulation which prevents rapid clearance and uptake by MPS (7). Moreover, the use of pegylated lipids with distal functional moieties such as maleimide allows the conjugation of targeting ligands to the liposomes. Targeted liposomes enhance the specificity and efficiency of treatment which may result in a higher therapeutic success rate and in reduction of undesirable side effects (8). An important requirement for successful targeted delivery is the specificity of the ligand/receptor pair for the site of interest. For example, we have previously demonstrated that a RGD-peptide functions as a targeting ligand for integrin $\alpha v \beta 3$ which can be used to target liposomes specifically to angiogenically activated endothelial cells (9).

Angiogenesis, i.e., the outgrowth of new vessels from pre-existing vasculature, is a prerequisite for tumor growth and metastasis (10, 11). The tumors' dependence on this process, together with the observation that the endothelial cells that mediate the vessel growth change the expression of specific cell surface-related molecules (12), makes tumor angiogenesis receptive for targeted treatment strategies. We have previously described the (33-mer) synthetic angiostatic peptide anginex that inhibits migration and proliferation of activated EC *in vitro* (13, 14) and exerts a strong antitumor response *in vivo* without

affecting physiological angiogenesis (15). It has been demonstrated that fluorescently labeled anginex specifically homes to tumor vasculature (15, 16). Recently, we identified galectin-1 as the main receptor of anginex on activated endothelial cells (17).

In the clinical oncology field there is a need for targeting ligands that allow more specific therapy and can be used for noninvasive imaging techniques (18). Using tumor angiogenesis markers for targeting, noninvasive imaging would allow more accurate diagnosis and monitoring of angiogenic development and the effect of angiostatic treatment (19, 20). On the basis of our observations, we hypothesized that anginex is an excellent ligand for targeting of therapeutic or diagnostic liposomes to activated endothelial cells in the tumor vasculature. To test this hypothesis, we conjugated anginex to fluorescently labeled and paramagnetic liposomes. The endothelial cell specificity of the anginex-liposome conjugates was confirmed on human umbilical vein endothelial cells (HUVEC) and an immortalized endothelial cell line (EVL-C2). In addition, we were able to demonstrate, by microscopic analysis and by MR imaging, that anginex-conjugated liposomes specifically bind to and are taken up by activated endothelial cells, making them attractive vehicles for targeted delivery or diagnostic use in the treatment of disease.

MATERIALS AND METHODS

Materials. 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cholesterol (Chol), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG2000-DSPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)2000] (Mal-PEG2000-DSPE), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (rhodamine-PE) were obtained from Avanti Polar Lipids (Albaster, AL). Gd-DTPA-bis-(stearylamine) (Gd-DTPA-BSA) was purchased from Gateway Chemical Technology (St. Louis, MO). HEPES was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytic grade or the best grade available. Polycarbonate filters for liposome extrusion were from Costar (Cambridge, MA).

* Corresponding author: Arjan W. Griffioen, Ph.D., Department of Pathology, Maastricht University & University Hospital Maastricht, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands, phone: +31-43-3874630, fax: +31-43-3876613, e-mail: aw.griffioen@path.unimaas.nl.

[#] Maastricht University & University Hospital.

[§] Eindhoven University of Technology.

[†] University of Minnesota.

Peptide Synthesis. Anginex with an additional cysteine at the N-terminal site was synthesized using a Milligen/Bioscience 9600 peptide solid-phase synthesizer using Fmoc chemistry. Lyophilized peptides were purified by preparative reversed-phase HPLC on a C18 column with an elution gradient of 0–60% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid in water. Purity and composition of the peptides were verified by HPLC (Beckman Model 6300) analysis of amino acid composition of hydrolysates prepared by treating the peptides under argon in 6 N HCl for 24 h at 110 °C. The amino acid sequences of peptides were confirmed by N-terminal sequencing and mass spectrometry. As described before, the cyclic 5-mer RGD peptide (c(RGDf(-S-acetylthioacetyl)K)) was synthesized at a purity of 95% by Ansynth Service BV (9).

Liposome Preparation. Liposomes were prepared by lipid film hydration. A mixture of the appropriate amounts of lipids (typically 100–200 μ mol of total lipid) was dissolved in chloroform/methanol 1:1 (v/v) and evaporated to dryness by rotary evaporation at 40 °C. For the paramagnetic PEG liposomes that were used to couple RGD or anginex peptides covalently to the distal end of the PEG chains, Gd-BSA, DSPC, cholesterol, PEG2000–DSPE, and Mal-PEG2000–DSPE were mixed at a molar ratio of 0.75/1.10/1.0/0.075/0.075. For fluorescence microscopy, 0.1 mol % of rhodamine–PE was added. The lipid film was subsequently hydrated in 3 mL of HEPES-buffered saline (HBS), containing 20 mM HEPES and 135 mM NaCl (pH 6.5). The resulting lipid dispersion was extruded sequentially six times through polycarbonate membrane filters with a pore diameter of 400 nm and subsequently 10 times through filters with a pore diameter of 200 nm using a Lipofast Extruder (Avestin, Canada). The temperature during extrusion was 65 °C.

Peptide Coupling to Liposomes. The cyclic 5mer RGD (c(RGDf(-S-acetylthioacetyl)K)) and anginex were conjugated to the liposomes by sulfhydryl-maleimide coupling maleimide-PEG–DSPE. Acetyl-protected RGD–peptide was deacetylated in 0.05 M HEPES/0.05 M hydroxylamine-HCl/0.03 mM ethylenediamine tetraacetic acid (pH 7.0) for 1 h at room temperature (21). The activated peptide was added to the Mal-PEG2000–DSPE-containing liposomes in a 3 μ g peptide/ μ mol lipid ratio. Anginex, synthesized with a C-terminal cysteine, was dissolved in water and added to the liposomal solution (10 μ g peptide/1 μ mol lipid). Coupling of both peptides was allowed overnight at 4 °C, and uncoupled peptide was separated from the liposomes by centrifuging at 200 000g with a Beckman Optima™ LE-80K ultracentrifuge and a Ti70 fixed angle rotor for 60 min. The liposomes were respectively conjugated with ~700 cyclic RGD moieties and ~125 anginex moieties per particle. The supernatant was removed, and an appropriate amount of buffer, typically 1.5 mL, was added to the pellet to obtain a lipid suspension with a lipid concentration of ~40 mM (10 mM gadolinium). The final liposomal suspension was stored at 4 °C under N₂.

Cell Culture. HUVECs were isolated as described before (22). Isolated cells were cultured in gelatin-coated culture flasks (Costar) with RPMI1640 (Gibco) supplemented with 20% human serum, 2 mM L-glutamine (Gibco) and 50 U/mL penicillin/streptomycin (Life sciences). Immortalized endothelial cell line EVL-C2 (23) was cultured under the same culture conditions as HUVEC. Cultures were incubated at 37 °C in the presence of 5% CO₂.

In Vitro Targeting of Endothelial Cells. For all tested applications the initial steps were similar. Endothelial cells were cultured in gelatin-coated flasks to approximately 75% confluency (approximately 1.5×10^6 cells) using standard culture conditions. Flasks were washed once with PBS before 4 mL of fresh culture medium was added. Anginex-conjugated, RGD-

conjugated, and nonconjugated liposomes (150 μ L of liposomes; total amount anginex 60 μ g) were added to the cells and incubated in the CO₂ incubator at 37 °C for 3 h. Subsequently, liposome-containing medium was removed, the cells were washed with 14 mL of PBS, and fresh medium was added. The final wash step was skipped for the control cells. Finally, the association of the targeted and nontargeted liposomes with endothelial cells was assessed under a phase contrast fluorescence microscope (Leica), at 20 \times and 40 \times magnification. Experiments were carried out in triplicate. Competition experiments were performed with free anginex. To that end, anginex (3 mg/mL in MQ) was added to the medium to a final concentration of respectively 10 or 30 μ g/mL (total anginex concentration respectively 60 and 180 μ g) immediately before addition of the liposome solution.

Magnetic Resonance Imaging of Cell Pellets. For MRI imaging, cells were harvested using trypsin digestion and centrifuged for 5 min at 400g. The obtained cell pellet was washed with approximately 5 mL of culture medium. After a second centrifugation step, the cell pellet was fixed with 1% paraformaldehyde and put in small 200 μ L tubes. MRI experiments were performed on fixed cell pellets using a 6.3 T horizontal bore magnet (Oxford Instruments Superconductivity, Eynsham, Oxon, England) interfaced to a Varian (Varian, Palo Alto, CA) VXR-S MRI console. A 3 cm quadrature-driven birdcage coil was used. The cups containing cell pellets were placed in a custom-made sample holder, capable of carrying four Eppendorf cups. Quantification of *T*₁ was done with an inversion recovery spin–echo sequence with 13 different inversion times, ranging from 10 to 3100 ms in an exponential fashion. The MRI data analysis was performed using Mathematica 5.0 (Wolfram Research, Inc., Champaign, IL). *T*₁ maps of HUVEC pellets were calculated in one slice through the pellet on a pixel-by-pixel basis. The *T*₁ is reported as mean \pm SD of the pixels in a circular region of interest (ROI) defined by the contour of the pellet. In Student's *t* tests, *P* < 0.05 was considered statistically significant.

RESULTS

We set out to test the applicability of anginex as a ligand to target liposomes to activated endothelial cells. To that end, fluorescent paramagnetic liposomes were prepared to allow parallel detection of the liposomes with optical methods as well as with MR imaging. Targeting specificity was introduced by covalently linking anginex containing a free cysteine via the thiol group to the maleimide group of the lipid Mal-PEG–DSPE (24). As a control, cyclic RGD was coupled as described previously (9). A schematic presentation of the ligand-conjugated liposomes is given in Figure 1.

To demonstrate specific binding to endothelial cells, cultured human umbilical vein endothelial cells (HUVEC) were incubated for 3 h with either anginex-conjugated liposomes or with bare liposomes. After liposome incubation and extensive washing, phase contrast microscopy revealed that only anginex-conjugated liposomes efficiently bound to the cell surface (data not shown). RGD-conjugated liposomes, used as a positive control, also bound the endothelial monolayer. Using fluorescence microscopy, the liposomes were visualized and were found to bind throughout the endothelial surface (Figure 2a, left and middle panel). Cells incubated with bare liposomes did marginally or did not show fluorescence signal (Figure 2a, right panel). Although small differences in morphology were visible between the three independent experiments due to different passage numbers, the binding or presence of the liposomes did not induce obvious changes in EC morphology or signs of apoptosis. At higher magnification, internalization of part of the conjugated liposomes was visible in vesicles (Figure 2b,

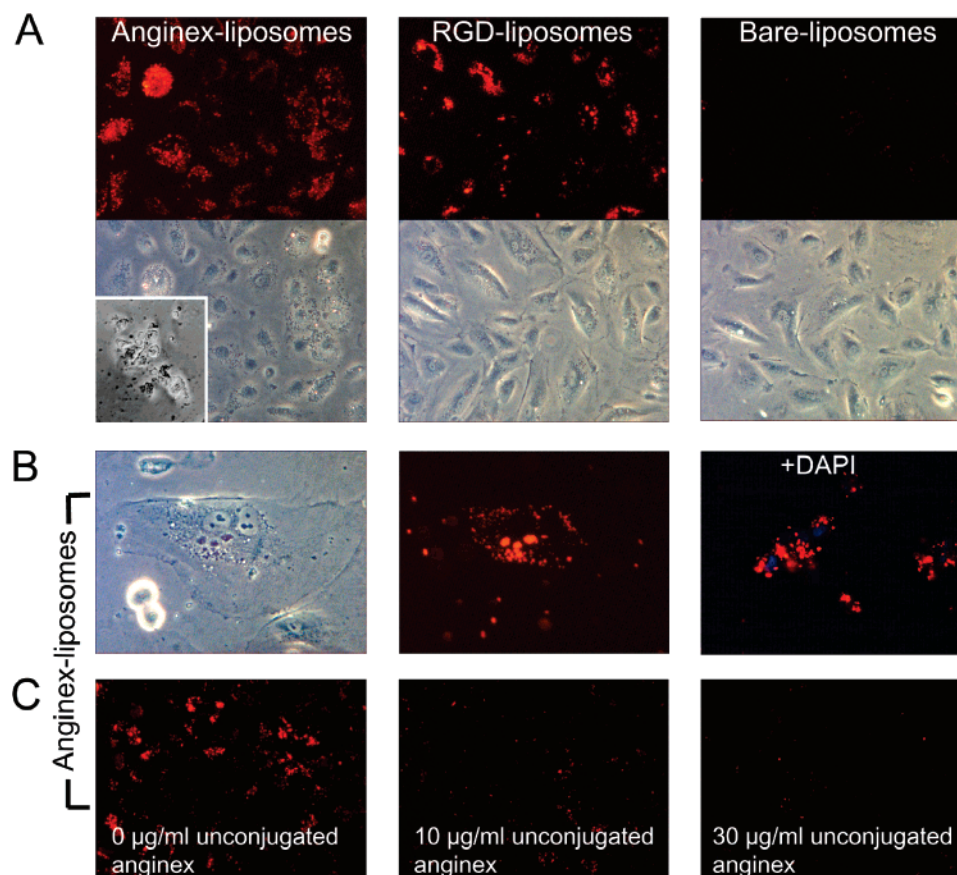


Figure 2. Fluorescence microscopy of HUVEC incubated with fluorescently labeled paramagnetic liposomes. Pictures are representative for all experiments. (A) HUVEC were incubated for 3 h with respectively Anginex, RGD, and nontargeted liposomes. Insert: HUVEC undergoing apoptosis as a result of Anginex treatment. (B) Perinuclear localization of Anginex–liposome conjugates in one HUVEC cell shown by phase contrast and fluorescence image. Anginex–liposome conjugates do not enter the nucleus as shown by blue DAPI counter staining. (C) Competition of Anginex–liposome conjugates with respectively 0, 10, 30 µg/mL free Anginex.

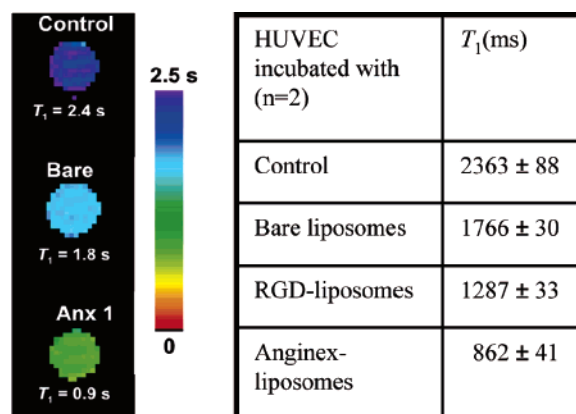


Figure 3. T_1 -weighted image of cells incubated with Anginex, bare liposomes, or without liposomes. The T_1 relaxation times of the different cell pellets are presented on the right.

would be sufficient to attain partial or complete blocking of Anginex–liposome binding. Ultimate proof for receptor-mediated uptake of the Anginex–liposome conjugates would be obtained in galectin-1 knockout endothelial cells. Indeed, tumors in galectin-1 knockout mice no longer respond to Anginex treatment, suggesting that the binding of the Anginex–liposome conjugates to HUVEC is Anginex- and galectin-1-dependent.

To evaluate if Anginex is also suitable as targeting ligand for MRI contrast agents, a quantitative T_1 -measurement was made of HUVEC pellets incubated with Anginex-conjugated paramagnetic liposomes. As a positive control, RGD–liposome

conjugates were used (9), and as negative control, bare paramagnetic liposomes or no liposomes were applied. Both conjugated paramagnetic liposomes were shown to bind specifically to endothelial cells. Furthermore, a clear difference in contrast was seen between ligand-conjugated liposomes and the controls leading to a significant difference in T_1 values. The fact that the conjugated liposomes were internalized might also be a benefit for imaging. By internalizing the contrast agent, the concentration per cell can be elevated, revealing a brighter signal (31).

Besides diagnostic applications, Anginex-targeted liposomes might be used as drug carrier. Therefore, water soluble drugs (e.g., doxorubicin) or even DNA constructs for gene therapy can be put inside the liposomal lumen (32, 33). Thus, Anginex-mediated targeting to tumor vasculature might be used to combine diagnosis and local therapy of cancer. However, for imaging, stabilization of liposomes is useful with drug delivery because the stability causes a slow release of the drugs. Another problem is the measurement of the amount of drugs released in the tissue. This can be solved by combining the drug and the contrast agent instead of loading drugs into liposomes with contrast-enhancing components in their bilayer (34, 35). Nevertheless, preliminary data with contrast-enhancing liposomes loaded with doxorubicin show promising results *in vivo* (unpublished data).

Anginex is a potent angiostatic agent, and one of the main angiostatic characteristics of Anginex is induction of apoptosis in activated EC. Although this might interfere with targeting, we have demonstrated that the induction of apoptosis by Anginex occurs more than 48 h after administration (13). This is far beyond the time frame of targeting, suggesting that the induction

of apoptosis will not affect targeting. This is supported by the fact that other vascular targeting ligands, such as RGD-peptides, also induce endothelial cell apoptosis (36–38) without affecting their applicability as a targeting ligand (39, 40).

Similar to that for apoptosis, the time frame of the experiments in this study did not allow us to investigate the effects of conjugated anginex on migration and proliferation (41). However, for imaging purposes, a possible effect will not influence the targeting ability and its function as a contrast agent.

In conclusion, we have demonstrated that the angiostatic peptide anginex is a potent ligand for the targeting of liposomes to activated endothelial cells. In addition, the *in vitro* results show a high specificity of the anginex-conjugated paramagnetic liposomes for endothelial cells that was confirmed by MRI. Therefore, anginex might serve as a targeting ligand for drug and gene therapy applications and for imaging of tumor vasculature in order to diagnose, treat, and monitor cancer.

ACKNOWLEDGMENT

This study was supported by a generous research grant from the Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affairs (MPG 5465 to A.W.G.)

LITERATURE CITED

- Brandwijk, R. J. M. G. E., Griffioen, A. W., and Thijssen, V. L. J. L. (2007) Targeted gene delivery strategies for angiostatic cancer treatment. *Trends Mol. Med.*, in press.
- Koning, G. A., Schiffelers, R. M., and Storm, G. (2002) Endothelial cells at inflammatory sites as target for therapeutic intervention. *Endothelium* 9, 161–71.
- Torchilin, V. P. (2005) Recent advances with liposomes as pharmaceutical carriers. *Nat. Rev. Drug Discovery* 4, 145–60.
- Lasic, D. (1993) *Liposomes: from physics to applications*, Chapter 11, Elsevier Science Publishing Company, Amsterdam.
- Papahadjopoulos, D., and Gabizon, A. (1990) Liposomes designed to avoid the reticuloendothelial system. *Prog. Clin. Biol. Res.* 343, 85–93.
- Allen, T. M. (1994) The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system. *Adv. Drug Delivery Rev.* 13, 285–309.
- Allen, T. M., and Hansen, C. (1991) Pharmacokinetics of stealth versus conventional liposomes: effect of dose. *Biochim. Biophys. Acta* 1068, 133–41.
- Backer, M. V., Aloise, R., Przekop, K., Stoletov, K., and Backer, J. M. (2002) Molecular vehicles for targeted drug delivery. *Bioconjugate Chem.* 13, 462–7.
- Mulder, W. J., Strijkers, G. J., Habets, J. W., Bleeker, E. J., van der Schaft, D. W., Storm, G., Koning, G. A., Griffioen, A. W., and Nicolay, K. (2005) MR molecular imaging and fluorescence microscopy for identification of activated tumor endothelium using a bimodal lipidic nanoparticle. *FASEB J.* 19, 2008–10.
- Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* 1, 27–31.
- Molema, G., and Griffioen, A. W. (1998) Rocking the foundations of solid tumor growth by attacking the tumor's blood supply. *Immunol. Today* 19, 392–4.
- Bergers, G., and Benjamin, L. E. (2003) Tumorigenesis and the angiogenic switch. *Nat. Rev. Cancer* 3, 401–10.
- Griffioen, A. W., van der Schaft, D. W., Barendsz-Janson, A. F., Cox, A., Struijker Boudier, H. A., Hillen, H. F., and Mayo, K. H. (2001) Anginex, a designed peptide that inhibits angiogenesis. *Biochem. J.* 354, 233–42.
- Dings, R. P., Arroyo, M. M., Lockwood, N. A., van Eijk, L. I., Haseman, J. R., Griffioen, A. W., and Mayo, K. H. (2003) Beta-sheet is the bioactive conformation of the anti-angiogenic anginex peptide. *Biochem. J.* 373, 281–8.
- van der Schaft, D. W., Dings, R. P., de Lussanet, Q. G., van Eijk, L. I., Nap, A. W., Beets-Tan, R. G., Bouma-Ter Steege, J. C., Wagstaff, J., Mayo, K. H., and Griffioen, A. W. (2002) The designer anti-angiogenic peptide anginex targets tumor endothelial cells and inhibits tumor growth in animal models. *FASEB J.* 16, 1991–3.
- Akerman, M. E., Pilch, J., Peters, D., and Ruoslahti, E. (2005) Angiostatic peptides use plasma fibronectin to home to angiogenic vasculature. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2040–5.
- Thijssen, V. L., Postel, R., Brandwijk, R. J., Dings, R. P., Nesmelova, I., Satijn, S. A., Verhofstad, N., Nakabeppu, Y., Baum, L. G., Bakkers, J., Mayo, K. H., Poirier, F., and Griffioen, A. W. (2006) Galectin-1 is essential in angiogenesis and is a target for anti-angiogenesis therapy. *Proc. Natl. Acad. Sci. U.S.A.* 103, 15975–15980.
- McDonald, D. M., and Choyke, P. L. (2003) Imaging of angiogenesis: from microscope to clinic. *Nat. Med.* 9, 713–25.
- Moseley, M. (2004) Multimodality Imaging: Introduction. *Stroke* 35, 2632–2634.
- Dobrov, M., Serganova, I., Mayer-Kuckuk, P., Ponomarev, V., and Blasberg, R. G. (2004) Multimodality in vivo molecular-genetic imaging. *Bioconjugate Chem.* 15, 1376–88.
- Kok, R. J., Schraa, A. J., and Bos, E. J. (2002) Preparation and functional evaluation of RGD-modified proteins as $\alpha_v\beta_3$ integrin directed therapeutics. *Bioconjugate Chem.* 13, 128–135.
- van der Schaft, D. W., Toebes, E. A., Haseman, J. R., Mayo, K. H., and Griffioen, A. W. (2000) Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells. *Blood* 96, 176–81.
- van Leeuwen, E. B., Veenstra, R., van Wijk, R., Molema, G., Hoekstra, A., Ruiters, M. H., and van der Meer, J. (2000) Characterization of immortalized human umbilical and iliac vein endothelial cell lines after transfection with SV40 large T-antigen. *Blood Coagul. Fibrinol.* 11, 15–25.
- Mulder, W. J., Strijkers, G. J., Griffioen, A. W., van Bloois, L., Molema, G., Storm, G., Koning, G. A., and Nicolay, K. (2004) A liposomal system for contrast-enhanced magnetic resonance imaging of molecular targets. *Bioconjugate Chem.* 15, 799–806.
- Brandwijk, R. J., Dings, R. P., van der Linden, E., Mayo, K. H., Thijssen, V. L., and Griffioen, A. W. (2006) Anti-angiogenesis and anti-tumor activity of recombinant anginex. *Biochem. Biophys. Res. Commun.* 349, 1073–8.
- Dings, R. P., van der Schaft, D. W., Hargittai, B., Haseman, J., Griffioen, A. W., and Mayo, K. H. (2003) Anti-tumor activity of the novel angiogenesis inhibitor anginex. *Cancer Lett.* 194, 55–66.
- Mayo, K. H., Dings, R. P., Flader, C., Nesmelova, I., Hargittai, B., van der Schaft, D. W., van Eijk, L. I., Walek, D., Haseman, J., Hoye, T. R., and Griffioen, A. W. (2003) Design of a partial peptide mimetic of anginex with antiangiogenic and anticancer activity. *J. Biol. Chem.* 278, 45746–52.
- Ruoslahti, E., and Pierschbacher, M. D. (1986) Arg-Gly-Asp: a versatile cell recognition signal. *Cell* 44, 517–8.
- Claussie, N., van den Brule, F., Waltregny, D., Garnier, F., and Castronovo, V. (1999) Galectin-1 expression in prostate tumor-associated capillary endothelial cells is increased by prostate carcinoma cells and modulates heterotypic cell-cell adhesion. *Angiogenesis* 3, 317–25.
- Lotan, R., Belloni, P. N., Tressler, R. J., Lotan, D., Xu, X. C., and Nicolson, G. L. (1994) Expression of galectins on microvessel endothelial cells and their involvement in tumour cell adhesion. *Glycoconjugate J.* 11, 462–8.
- van Tilborg, G. A., Mulder, W. J., Deckers, N., Storm, G., Reutelingsperger, C. P., Strijkers, G. J., and Nicolay, K. (2006) Annexin A5-functionalized bimodal lipid-based contrast agents for the detection of apoptosis. *Bioconjugate Chem.* 17, 741–9.
- Allen, T. M. (1997) Liposomes. Opportunities in drug delivery. *Drugs* 54 Suppl 4, 8–14.
- Drummond, D. C., Meyer, O., Hong, K., Kirpotin, D. B., and Papahadjopoulos, D. (1999) Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. *Pharmacol. Rev.* 51, 691–743.
- Ponce, A. M., Viglianti, B. L., Yu, D., Yarmolenko, P. S., Michelich, C. R., Woo, J., Bally, M. B., and Dewhirst, M. W. (2007) Magnetic resonance imaging of temperature-sensitive liposome release: drug dose painting and antitumor effects. *J. Natl. Cancer Inst.* 99, 53–63.
- Viglianti, B. L., Ponce, A. M., Michelich, C. R., Yu, D., Abraham, S. A., Sanders, L., Yarmolenko, P. S., Schroeder, T., MacFall, J.

- R., Barboriak, D. P., Colvin, O. M., Bally, M. B., and Dewhirst, M. W. (2006) Chemodosimetry of in vivo tumor liposomal drug concentration using MRI. *Magn. Reson. Med.* 56, 1011–8.
- (36) Meerovitch, K., Bergeron, F., Leblond, L., Grouix, B., Poirier, C., Bubenik, M., Chan, L., Gourdeau, H., Bowlin, T., and Attardo, G. (2003) A novel RGD antagonist that targets both $\alpha_5\beta_3$ and $\alpha_5\beta_1$ induces apoptosis of angiogenic endothelial cells on type I collagen. *Vascul. Pharmacol.* 40, 77–89.
- (37) Modlich, U., Kaup, F. J., and Augustin, H. G. (1996) Cyclic angiogenesis and blood vessel regression in the ovary: blood vessel regression during luteolysis involves endothelial cell detachment and vessel occlusion. *Lab. Invest.* 74, 771–80.
- (38) Westlin, W. F. (2001) Integrins as targets of angiogenesis inhibition. *Cancer J.* 7 Suppl 3, S139–43.
- (39) Meyer, A., Auernheimer, J., Modlinger, A., and Kessler, H. (2006) Targeting RGD recognizing integrins: drug development, biomaterial research, tumor imaging and targeting. *Curr. Pharm. Des.* 12, 2723–47.
- (40) Temming, K., Schiffelers, R. M., Molema, G., and Kok, R. J. (2005) RGD-based strategies for selective delivery of therapeutics and imaging agents to the tumour vasculature. *Drug Resist. Updates* 8, 381–402.
- (41) Brandwijk, R. J., Nesmelova, I., Dings, R. P., Mayo, K. H., Thijssen, V. L., and Griffioen, A. W. (2005) Cloning an artificial gene encoding angiostatic anginex: From designed peptide to functional recombinant protein. *Biochem. Biophys. Res. Commun.* 333, 1261–8.

BC060316H