Acta Biochimica Polonica

Vol. 53 No. 4/2006, 801–805

on-line at: www.actabp.pl

Regular paper

Antitumor effect of RGD-4C-GG-$_D$(KLAKLAK)$_2$ peptide in mouse B16(F10) melanoma model

Ryszard Smolarczyk, Tomasz Cichoń, Klaudyna Graja, Joanna Hucz, Aleksander Sochanik and Stanisław Szala✉

Department of Molecular Biology, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice, Poland; ✉e-mail: sszala@io.gliwice.pl

Received: 29 June, 2006; revised: 12 October, 2006; accepted: 23 November, 2006
available on-line: 01 December, 2006

Vasculature targeting agents have been tested as cancer therapeutics for the past few years. Such therapy could be accomplished using, for example, bifunctional (two-domain) peptides. RGD-4C-GG-$_D$(KLAKLAK)$_2$, a peptide designed by Ellerby and coworkers (1999) (full sequence: ACDCRGDCFCGGKLAKLAKKLAKLAK), binds selectively to $\alpha_V\beta_3$ integrin receptors expressed in tumor neovasculature and, after internalization, effectively induces apoptosis of endothelial cells. The aim of this study was to examine if RGD-4C-GG-$_D$(KLAKLAK)$_2$ would efficiently target cells, among them B16(F10), that overexpress $\alpha_V\beta_3$ receptors, and whether it would be suitable for therapeutic treatment of primary B16(F10) murine melanoma tumors. Thus, the peptide would target two distinct tumor compartments: that formed by endothelium of blood vessels and that made up of neoplastic cells. The therapeutic peptide was recognized and did induce apoptosis in B16(F10) cell line. Tumor growth inhibition was observed following direct intratumoral administration. However, cessation of peptide administration led to rapid tumor growth and death of the animals.

Keywords: antivascular therapy, anticancer therapy, two-domain peptides

INTRODUCTION

Drugs acting specifically upon tumor vasculature are promising agents capable of destroying tumor blood vessels (for a review see: Szala, 2004; Thorpe, 2004; Tozer *et al*., 2005; Neri & Bicknell 2005). Such drugs have the so-called recognition domain responsible for identifying suitable markers on the luminal surface of tumor blood vessels and an effector domain mediating the therapeutic effect.

The recognition domain of these drugs is usually developed from antibodies that are identifiable by specific surface markers present on endothelial cells. Such markers include, for instance, endoglin, VCAM-1, E-selectin, or even extracellular matrix proteins (e.g. ED-B domain-containing fibronectin). The recognition domain of these drugs can also be formed from growth factor receptor (VEGFR) ligands or integrin receptor ($\alpha_V\beta_3$) ligands. The effector domain, in turn, can be formed from proteins, peptides and other chemical entities able to mediate apoptotic cell death (Kamysz *et al*., 2003; Szala, 2004; Thorpe, 2004; Tozer *et al*., 2005; Neri & Bicknell, 2005).

RGD-4C-GG-$_D$(KLAKLAK)$_2$, a peptide synthesized by Ellerby *et al*. (1999), is a simple example of the therapeutic agents in question. Its full amino-acid sequence is: ACDCRGDCFCGGKLAKLAKKLAKLAK. The first ten amino acids form the recognition domain (ACDCRGDCFC, abbreviated as RGD-4C) which is a ligand for $\alpha_V\beta_3$ integrin receptor; the two glycines provide a linker element (GG), whereas $_D$(KLAKLAK)$_2$, a proapoptotic peptide composed of D-amino acids, forms the effector domain. This drug effectively inhibited growth of MDA-MB-435 human breast carcinoma tumors (Ellerby *et al*., 1999).

Abbreviations: DTT, dithiothreitol; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phopshate-buffered saline; TMR, tetramethylrhodamine; VEGFR, vascular endothelial growth factor receptor.

The goal of our study was to examine whether RGD-4C-GG-$_D$(KLAKLAK)$_2$ was suitable for therapy of experimental B16(F10) primary melanoma tumors in mice. In particular, we wanted to check whether this drug would indeed show an increased therapeutic effect as a result of its twofold mode of action: acting upon tumor blood vessels and also eliminating cancer cells.

MATERIALS AND METHODS

Peptides: RGD-4C, $_D$(KLAKLAK)$_2$ and RGD-4C-GG-$_D$(KLAKLAK)$_2$. Throughout the experiments the therapeutic peptide RGD-4C-GG-$_D$(KLAKLAK)$_2$, as well as control peptides RGD-4C and $_D$(KLAKLAK)$_2$, were used. They were synthesized by GeneScript (Piscataway, NJ, USA).

Cell culture. The following cell lines were used: B16(F10), MDA-MB-435, HeLa, C26 and MCF-7 (all from ATCC); Heca10 (lymph node-derived and provided by Dr. D. Duś, Institute of Immunology and Experimental Therapy, Wrocław, Poland). Cells were cultured using RMPI 1640 media supplemented with 10% FBS and using NUNCLON™ Surface (NUNC™) culture vessels. Cultures were kept in a humidified standard incubator (37°C and 5% CO_2) (Mitrus *et al*., 2006).

Identification of β_3 integrin in cell lines by Western blotting. B16(F10), Heca10 and MCF-7 cell cultures were incubated overnight in 10-cm dishes. The cells were rinsed with PBS$^-$ (pH 7.2) and lysed using lysis buffer (2% SDS; 50 mM DTT; 62.5 mM Tris (pH 6.8); 0.01% bromophenol blue). Cell lysates were sonicated 10–15 s, denatured for 5 min/100°C and centrifuged. Proteins were separated on 8% polyacrylamide gels. Prestained Protein Ladder and Protein Ladder (MBI Fermentas, Lithuania) were used as molecular mass markers. Separated proteins were electrotransferred onto nitrocellulose membranes (1.5 h, 4°C, 100 V constant voltage) which were then stained for 3 min using a solution (0.2% Ponceau Red and 6% trichloroacetic acid), washed in deionized water, and destained with TBST buffer (150 mM NaCl; 0.05% Tween-20; 10 mM Tris/HCl (pH 7.4)). For immunohistochemical detection we used polyclonal rabbit (first) antibody (Cell Signaling) recognizing integrin β_3 subunit and anti-rabbit horseradish peroxidase-conjugated goat (second) antibody (Vector). The membranes were incubated for 2 h, room temp. using the first antibody (1:1000 dilution) in TBS buffer and 3% milk. Incubation with the second antibody (1:500 dilution) in TBS and 1% milk was performed for 1.5 h, room temp. The immunoreactive proteins were visualized by chemiluminescence (Super Signal West Substrate kit, Pierce). For quantitative assessment, antibodies were stripped off with a solution (2% SDS; 100 mM β-mercaptoethanol; 65.5 mM Tris/HCl (pH 6.2)); the membrane was then incubated with rabbit anti-β actin antibody (Cell Signaling).

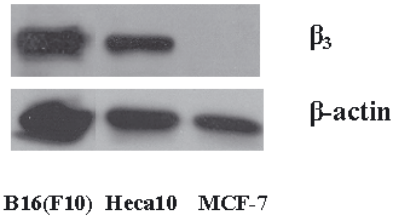

Figure 1. Western blot determination of β_3 domain in various cell lines.
Heca10, B16(F10) or MCF-7 cell cultures were lysed. Gel-separated proteins were transferred onto nitrocellulose membrane. Rabbit polyclonal antibody (Sigma) recognizing β-3 integrins and anti-rabbit goat horseradish peroxidase-conjugated antibody (Vector) were used. Proteins were visualized using Super Signal West Substrate (Pierce) chemiluminescence kit. Following removal of membrane-bound antibodies the membrane was incubated with rabbit anti-β-actin antibody (Sigma).

Peptide cytotoxicity. Cells were grown in 96-well plates (NUNCLON™ Surface, 2 × 10^3 cells per well, 100 μL of RMPI 1640 supplemented with 10% FBS). After 24 h, one of the two control peptides or the therapeutic peptide were added in quadruplicate to the culture media using eleven different concentrations (2–200 μM, see Fig. 2). The culture vessels were then placed in a 5% CO_2/37°C incubator for further 24 h. Next, culture medium was replaced with MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] solution (0.5 mg MTT/1 mL PBS$^-$) and the cells were additionally incubated for 3 h at 37°C. The formazan crystals formed were dissolved in acidic isopropanol. Spectrophotometric measurements were performed at λ = 570 nm using an ELISA EL$_x$800 reader (Bio-Tek Instruments, Inc.). The percentage of live cells was

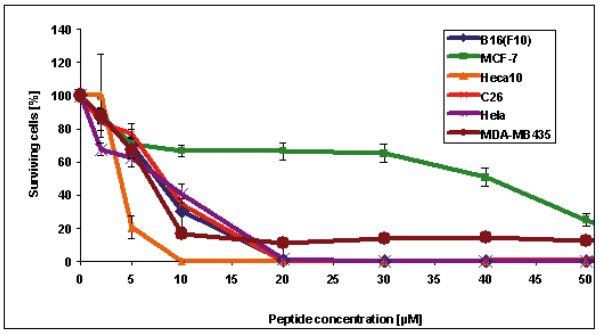

Figure 2. Cytotoxicity of RGD-4C-GG-$_D$(KLAKLAK)$_2$ peptide in various cell lines.
Cells were cultured using 96-well plates. In each well 2000 cells were seeded in 100 μL medium. Following 24-h incubation culture medium was replaced with medium containing appropriate concentrations of RGD-4C-GG-$_D$(KLAKLAK)$_2$ therapeutic peptide and the cultures were incubated for further 24 h. The experiment was concluded with MTT test determining the number of live cells.

estimated as: (absorbance at time t/initial absorbance) × 100%.

TUNEL assay. Cells (2×10^4) suspended in 250 μL medium were seeded in gelatin-covered 8-well Chamber Slide plates. After 24 h either the therapeutic or control peptides (final conc. 10 μM) were added to the media. The cell cultures were kept in a 5% CO_2 at 37°C incubator for additional 3 h.

Cells undergoing apoptosis were visualized using the In Situ Cell Death Detection Kit, TMR red (Roche), which stains nuclei with fragmented DNA. The stained cells were photographed (λ = 540 nm, Nicon Eclipse 80i fluorescence microscope equipped with Lucia software).

Effect of RGD-4C-GG-$_D$(KLAKLAK)$_2$ on growth of melanoma tumors. All procedures involving animals were performed with the approval of the Local Ethics Committee, Medical Academy, Warsaw, Poland. Therapy was performed using 6 to 8-week-old C57BL/6 mice from own animal facility. Each animal was injected intradermally (dorsal side) with 2×10^5 B16(F10) cells/100 μL PBS^- (pH 7.2). When tumors reached about 3 × 3 mm (on the 6th day), mice received the therapeutic RGD-4C-GG-$_D$(KLAKLAK)$_2$ peptide intravenously for two weeks (50 μg, 100 μg or 250 μg, see Fig. 4) or intratumorally (900 μM, see Fig. 5). Additionally, control RGD-4C peptide (900 μM) was administered intratumorally (for all experimental details and schedule see Figs. 4 and 5). Tumor size (caliper-measured), tumor growth rate as well as survival of animals were monitored.

RESULTS AND DISCUSSION

RGD-4C-GG-$_D$(KLAKLAK)$_2$, a therapeutic peptide, acts upon endothelial cells which express integrin $\alpha_V\beta_3$ surface receptors (Ellerby *et al.*, 1999). Such receptors are found not only on the luminal side of tumor endothelium but are also expressed in some types of cancer cells, for example B16(F10) murine melanoma (Zitzmann *et al.*, 2002; Line *et al.*, 2005; Mitra *et al.*, 2005). Using antibodies directed against β_3 subunit of the receptor we have shown that B16(F10) as well as endothelial Heca10 cell lines indeed express this subunit, whereas the MCF-7 cell line (negative control) does not (Fig. 1). Since B16(F10) cells express the $\alpha_V\beta_3$ receptor we decided to investigate whether the RGD-4C-GG-$_D$(KLAKLAK)$_2$ therapeutic peptide would be useful for treating this type of murine melanoma tumors. We expected to observe results of the twofold mode of action of this drug. The augmented therapeutic

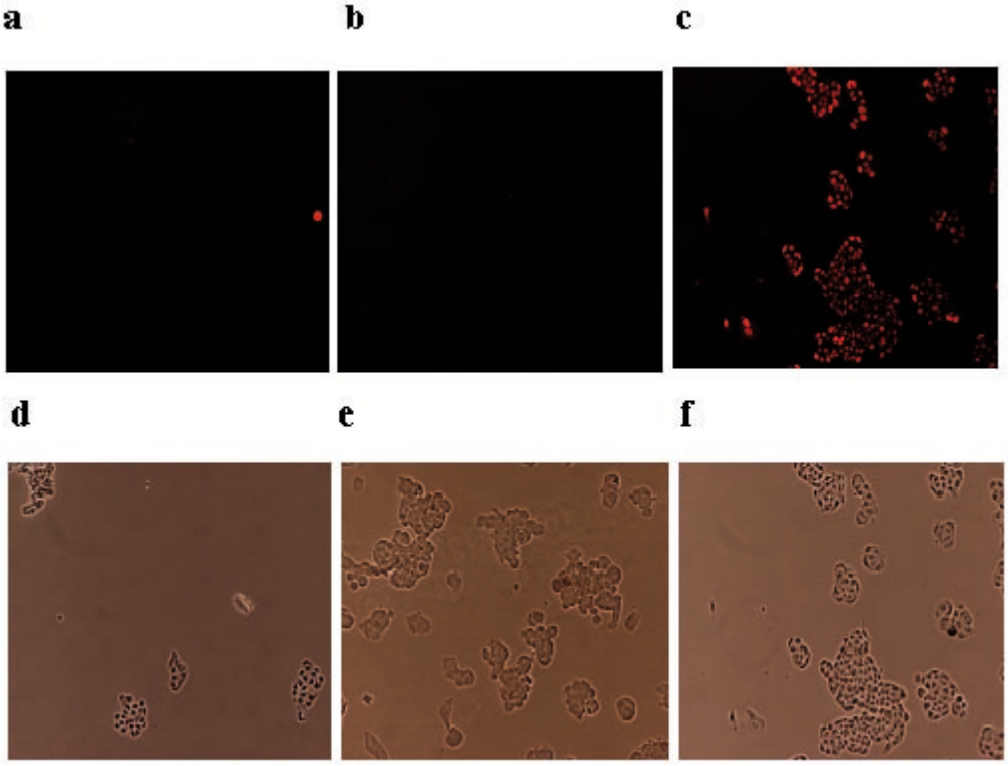

Figure 3. Apoptosis in B16(F10) cells.
Gelatin-covered Chamber Slide plates were seeded with 2×10^4 B16(F10) cells per well using 250 μL medium. After 24-h incubation control peptides: RGD-4C (a, d), $_D$(KLAKLAK)$_2$ (b, e) or the therapeutic peptide RGD-4C-GG-$_D$(KLAKLAK)$_2$ (c, f) were added to the wells (final concentration 10 μM) in 250 μL medium. Cultures were incubated for further 3 h at 37°C. Apoptosis was determined using TUNEL test. Stained cells were viewed after 24 h using a Nicon Eclipse 80i fluorescence microscope at 540 nm. Photographs were taken using Lucia software.

effect was expected to result first from the drug acting upon tumor blood vessels and, second, from it eliminating cancer cells.

Using the MTT test we determined the specificity of this peptide towards the used cell lines expressing the $\alpha_V\beta_3$ receptor. We demonstrated that the peptide is highly toxic for the following cell lines: B16(F10), Heca10, MDA-MB-435, C26 and HeLa, whereas for MCF-7 cells, devoid of $\alpha_V\beta_3$ receptors, it is much less so (Fig. 2). For the cell lines expressing $\alpha_V\beta_3$ (B16(F10), MDA-MB-435, C26 and HeLa) the LC_{50} values of the peptide were about 7 μM, and for Heca10 about 3 μM. For the cell line devoid of $\alpha_V\beta_3$ receptors the LC_{50} was much higher, about 45 μM. Both control peptides, RGD-4C and $_D$(KLAKLAK)$_2$, proved nontoxic for the examined cell lines (not shown).

The investigated RGD-4C-GG-$_D$(KLAKLAK)$_2$ peptide, when internalized, causes cell apoptosis (Ellerby *et al*., 1999). We confirm this observation. TUNEL test showed that in nuclei of B16(F10) cells treated with this peptide DNA was fragmented as visualized using fluorescence microscopy (Fig. 3). On the other hand, B16(F10) cells treated with the control peptides and MCF-7 cells treated with either the therapeutic or one of the control peptides did not produce red nuclear fluorescence. This means a lack of DNA fragmentation and apoptosis in those cells (Fig. 3).

Following internalization, the proapoptotic $_D$(KLAKLAK)$_2$ peptide acts by breaking down mitochondrial membrane, which causes cytochrome *c* efflux into the cytoplasm (Ellerby *et al*., 1999). Once there, cytochrome *c* forms complexes with Apaf-1 and caspase 9 proenzyme. Activation of this caspase triggers irreversible conversion cascade of other caspases, leading to apoptosis of endothelial cells.

We further attempted therapy of primary B16(F10) melanoma tumors in mice by employing the RGD-4C-GG-$_D$(KLAKLAK)$_2$ peptide supposed to target two separate tumor compartments formed by vasculature endothelium and neoplastic cells, respectively. The peptide was administered intravenously at varying doses (50, 100 and 250 μg). However, none of them caused significant inhibition of tumor growth (Fig. 4). Since high levels of $\alpha_V\beta_3$ receptor were observed, besides tumor endothelium, also in kidneys, liver and spleen, the peptide was probably sequestered during passage through these organs and did not reach its target site at a sufficient concentration (Line *et al*., 2005; Mitra *et al*., 2005). It is unlikely that the peptide was serum-inactivated since it is composed of D-amino acids (Ellerby *et al*., 1999).

The sequestration issue appears important in explaining the results of the study performed by Ellerby and coworkers which demonstrated inhibition of tumor growth following intravenous administration of RGD-4C-GG-$_D$(KLAKLAK)$_2$ (Ellerby *et al*., 1999) in experimental MDA-MB-435 human breast tumors grown in nude mice. This particular malignancy is characterized by slow rate of growth, so even small doses of the peptide were probably sufficient to inhibit tumor development. In the case of B16(F10) murine melanoma tumor model, characterized by a much more rapid growth rate, the sequestration of the administered peptide results in insufficient peptide supply at the tumor site.

On the other hand, when the peptides were injected directly into the primary tumors, the rate of growth was slower when the therapeutic peptide was administered, as compared to the control peptides (Fig. 5). On the 18th day of therapy a 94% in-

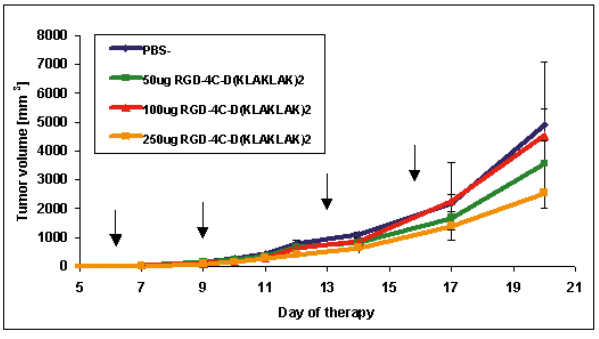

Figure 4. Kinetics of B16(F10) murine melanoma tumor growth following intravenous administration of RGD-4C-GG-$_D$(KLAKLAK)$_2$.
C57BL/6 mice were injected intradermally (dorsal side) with 2 × 10^5 B16(F10) cells. Starting on the 6th day following inoculation, animals were administered intravenously the therapeutic peptide (50 μg, 100 μg or 250 μg in 200 μL PBS^-, pH 7.2). As a control, 200 μL PBS^- (pH 7.2) was injected. The injections were administered (see arrows) twice a week for two weeks.

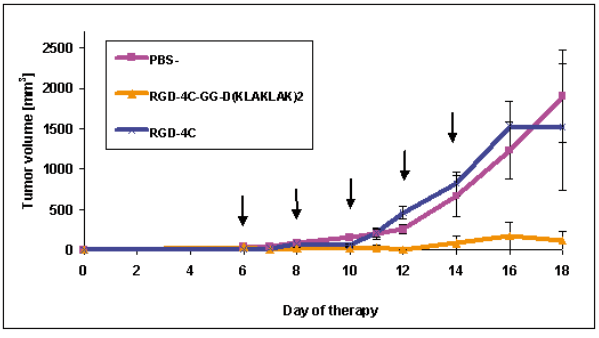

Figure 5. Kinetics of B16(F10) murine melanoma tumor growth following intratumoral administration of RGD-4C-GG-$_D$(KLAKLAK)$_2$ or RGD-4C peptides.
C57BL/6 mice were injected intradermally (dorsal side) with 2 × 10^5 B16(F10) cells. Starting on the 6th day following inoculation, 100 μL of peptide solution (900 μM) was administered intratumorally (RGD-4C-$_D$(KLAKLAK)$_2$ or RGD-4C) in PBS^- (pH 7.2). As a control, 100 μL PBS^- (pH 7.2) was injected. The injections were administered (see arrows) five times every two days.

hibition was noted with respect to animals injected PBS^- alone.

Based on the results obtained we were unable to discern the tumor antiangiogenic effect of the peptide used from its direct impact upon B16(F10) cells. It is tempting to speculate that intratumoral administration of RGD-4C-GG-$_D$(KLAKLAK)$_2$ results in destruction of both angiogenic tumor endothelial cells as well as neoplastic cells, since both express $\alpha_V\beta_3$ surface receptors. However, the therapeutic effect observed by us in murine melanoma tumors was so rapid that it proved difficult to demonstrate solely the antivascular effect of the peptide used. It seems that discrimination between the two mechanisms of the peptide's action might be achieved if similar experiments are carried out in nude mice using a tumor model featuring $\alpha_V\beta_3$ receptor-lacking cancer cells.

Cessation of therapeutic peptide administration led to rapid tumor growth and death of the animals (not shown). Probably, this relapse was brought about by some neoplastic cells that survived therapy. It is known that tumor architecture, especially its vascular network component, as well as other factors, notably intratumoral pressure etc., may cause restricted drug penetration into tumor mass, thereby giving rise to a pool of surviving cancer cells (Jain, 1994; 2005). In the absence of the drug, these cells are likely to cause tumor regrowth. For our therapy to be effective the peptide would have to be administered continuously; cessation inevitably leads to quick remission.

It appears reasonable to attempt tumor-targeted therapies based on similar peptide constructs and backed up by other modalities used to eliminate cancer cells, such as radiotherapy or novel chemotherapeutics. Combined therapies of this kind might perhaps offer the much sought solution allowing total eradication of cancer cells in solid tumors, thereby preventing their regrowth and metastases.

Acknowledgements

This study was supported by a grant from the State Committee for Scientific Research (KBN) No. 2 P05A 074 28.

REFERENCES

Ellerby HM, Arap W, Ellerby LM, Kain R, Andrusiak R, Rio GD, Krajewski S, Lombardo CR, Rao R, Ruoslahti E, Bredesen DE, Pasqualini R (1999) Anti-cancer activity of targeted pro-apoptotic peptides. *Nat Med* **5:** 1032–1038.

Jain RK (1994) Barriers to drug delivery in solid tumors. *Sci Am* **271:** 58–65.

Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* **307:** 58–62.

Kamysz W, Okroj M, Lukasiak J (2003) Novel properties of antimicrobial peptides. *Acta Biochim Polon* **50**: 461–469.

Line BR, Mitra A, Nan A, Ghandehari H (2005) Targeting tumor angiogenesis: comparison of peptide and polymer–peptide conjugates. *J Nucl Med* **46:** 1552–1560.

Mitra A, Mulholland J, Nan A, McNeill E, Ghandehari H, Line BR (2005) Targeting tumor angiogenic vasculature using polymer–RGD conjugates. *J Control Release* **102:** 191–201.

Mitrus I, Delic K, Wrobel N, Missol-Kolka E, Szala S (2006) Combination of IL–12 gene therapy and CTX chemotherapy inhibits growth of primary B16(F10) melanoma tumors in mice. *Acta Biochim Polon* **53**: 357–360.

Neri D, Bicknell R (2005) Tumour vascular targeting. *Nat Rev Cancer* **5:** 436–446.

Szala S (2004) Two-domain vascular disruptive agents in cancer therapy. *Curr Cancer Drug Targets* **4:** 501–509.

Thorpe PE (2004) Vascular targeting agents as cancer therapeutics. *Clin Cancer Res* **10:** 415–427.

Tozer GM, Kanthou C, Baguley BC (2005) Disrupting tumour blood vessels. *Nat Rev Cancer* **5:** 423–435.

Zitzmann S, Ehemann V, Schwab M (2002) Arginine-glycine-aspartic acid (RGD)-peptide binds to both tumor and tumor-endothelial cells *in vivo*. *Cancer Res* **62:** 5139–5143.