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RGD-based strategies for selective delivery of therapeutics and imaging agents to the tumour vasculature

Kai Temming [a,b], Raymond M. Schiffelers [c], Grietje Molema [d], Robbert J. Kok [a,*]

[a] *Department of Pharmacokinetics and Drug Delivery, Groningen University Institute for Drug Exploration (GUIDE), Antonius Deusinglaan 1, 9713 AV, Groningen, The Netherlands*
[b] *KREATECH Biotechnology B.V., Amsterdam, The Netherlands*
[c] *Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht, The Netherlands*
[d] *Department of Pathology and Laboratory Medicine, Medical Biology Section, University Medical Center Groningen, University of Groningen, The Netherlands*

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Abstract

During the past decade, RGD-peptides have become a popular tool for the targeting of drugs and imaging agents to $\alpha_v\beta_3$-integrin expressing tumour vasculature. RGD-peptides have been introduced by recombinant means into therapeutic proteins and viruses. Chemical means have been applied to couple RGD-peptides and RGD-mimetics to liposomes, polymers, peptides, small molecule drugs and radiotracers. Some of these products show impressive results in preclinical animal models and a RGD targeted radiotracer has already successfully been tested in humans for the visualization of $\alpha_v\beta_3$-integrin, which demonstrates the feasibility of this approach.

This review will summarize the structural requirements for RGD-peptides and RGD-mimetics as ligands for $\alpha_v\beta_3$. We will show how they have been introduced in the various types of constructs by chemical and recombinant techniques. The importance of multivalent RGD-constructs for high affinity binding and internalization will be highlighted. Furthermore the in vitro and in vivo efficacy of RGD-targeted therapeutics and diagnostics reported in recent years will be reviewed.
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1. Introduction

When the arginine-glycine-aspartic acid (RGD) sequence was discovered as cell attachment site in fibronectin some 20 years ago, it was unexpected that a fundamental recognition site for cells and proteins could be formed by only three amino acids (Pierschbacher and Ruoslahti, 1984; Ruoslahti, 2003). Soon thereafter, RGD-recognition sites were reported in other extracellular matrix (ECM) proteins as well (Grant et al., 1989; Lawler et al., 1988; Miyauchi et al., 1991; Pytela, 1988). In parallel, the receptors for these ECM proteins were identified and organized in the integrin family.

Integrins and RGD-based ligands for integrins are currently an intensely investigated topic in pathology and pharmacology related studies. Apart from binding natural RGD-containing ligands such as ECM proteins and counter-receptors on cells, RGD-recognition is also exploited by viruses and bacteria (Bergelson et al., 1992; Leininger et al., 1991; Logan et al., 1993; Wickham et al., 1993). These pathogens gain entry into host cells by binding to specific integrins and subsequent endocytosis. Furthermore, RGD-motifs and other integrin-binding sites have been found in snake venoms, enabling them to affect for instance blood coagulation (Calvete, 2005; Knudsen et al., 1988; Marcinkiewicz, 2005). Exploitation of the RGD/integrin system for target cell recognition and internalization can also be applied to man-made constructs by mimicking the pathogens. This enables the targeting of diagnostics and therapeutics.

* Corresponding author. Tel.: +31 50 363 3276; fax: +31 50 363 3247.
E-mail address: r.j.kok@rug.nl (R.J. Kok).

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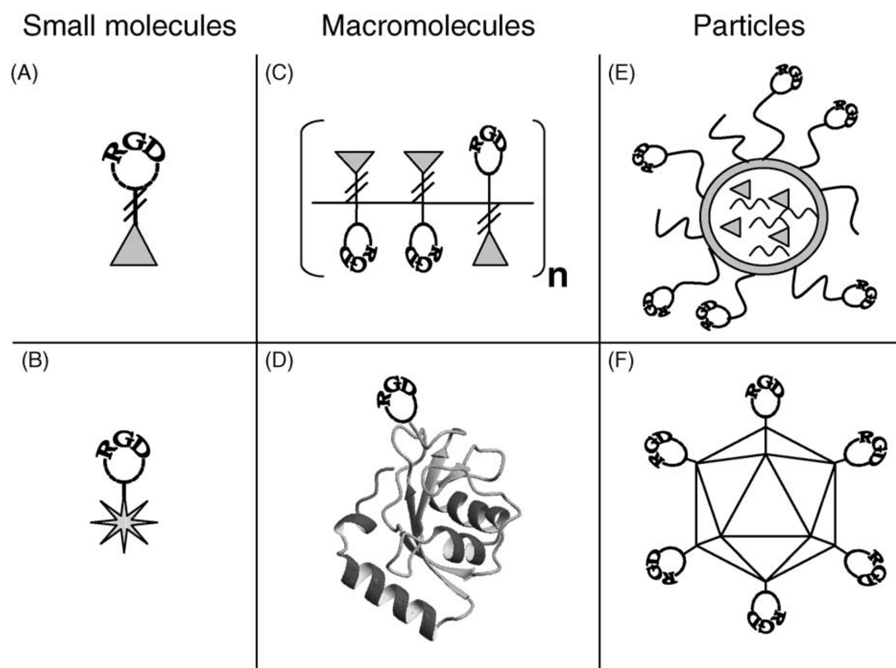

Fig. 1. Schematic representation of RGD-mediated drug delivery and imaging constructs. (Panels A and B) Small molecule conjugates. Organic drug molecules (A) have been conjugated to cyclic RGD-ligands via various linkers. As most drugs are inactive when coupled to the RGD-ligand, a biodegradable linkage is required between drug and RGD-peptide. In contrast, imaging agents (B) need stable linkages. (Panels C and D) Macromolecules. Synthetic and natural polymers (C) can be modified with multiple copies of both drug (affording a high drug/carrier ratio) and RGD (affording multivalent binding and internalization). Therapeutic proteins (D) have been equipped with RGD-motifs by recombinant means. (Panels E and F) Particulate systems. Synthetic particulate systems (E) like liposomes, nanoparticles or non-viral gene vectors have been equipped with RGD mostly via extended PEG tethers, to prevent unwanted interactions with non-target cells. Adenoviral vectors (F) have been modified with RGD motifs to enhance infectivity. Viral carriers have been used to deliver genes and siRNA, or as oncolytic replicating systems.

Typical examples of RGD-equipped constructs that will be discussed in this review are shown in Fig. 1. RGD-targeted drugs and imaging agents have been developed by covalent conjugation of the homing peptide to drug or reporter molecules (A and B), or by conjugation of the RGD-peptide to a carrier device that has been equipped with drug molecules (C and E). Similarly, RGD-peptides have been applied for gene delivery by viral and non-viral vectors (E and F). Another type of products that will be discussed are therapeutic proteins that have been modified with RGD targeting motifs. This is preceded by an overview of the molecular requirements of the RGD-ligand for targeting to $\alpha_v\beta_3$ integrin.

2. Requirements of RGD-peptide as an $\alpha_v\beta_3$-ligand

The RGD sequence is currently the basic module for a variety of RGD-containing peptides which display preferential binding to either $\alpha_v\beta_3$ integrin and related α_v-integrins, or to other types of integrins. For example, $\alpha_{IIb}\beta_3$ integrin is an integrin that has been investigated intensely in relation to platelet aggregation (Andronati et al., 2004). Since the RGD-sequence is conserved in all natural and new developed ligands, the relative affinity and specificity of the peptides and proteins are determined by other amino acid residues flanking the RGD-motif, especially at the two positions following the aspartic acid (Pierschbacher and Ruoslahti, 1987). Besides direct interactions between these residues and the integrin, flanking groups influence the folding of the peptide and thereby the conformational features of the RGD-motif. Cyclization is commonly employed to improve the binding properties of RGD-peptides. Since cyclization confers rigidity to structure, it greatly improves the selectivity of the promiscuous RGD-sequence for a specific integrin subtype. Indeed, all selective RGD-peptide ligands are cyclic, having at least one or more ring structures, as will be discussed. Furthermore, linear RGD-peptides proved highly susceptible to chemical degradation, which is due to the reaction of the aspartic acid residue (D) with the peptide backbone (Bogdanowich-Knipp et al., 1999). Since the rigidity conferred by cyclization prevents this, cyclic peptides are more stable. Lastly, non-natural peptide modifications such as the introduction of D-amino acids as well as replacement with peptidomimetic structures have yielded RGD-peptide ligands with increased specificity and nanomolar or higher affinity (Goodman et al., 2002). The elucidation of the crystal structure of $\alpha_v\beta_3$ integrin and subsequent docking studies on this template have aided in the design of novel RGD-ligands (Marinelli et al., 2003).

One of the best studied RGD-peptide ligands for $\alpha_v\beta_3$ integrin is c(RGDf-N(Me)-V), which is also known as

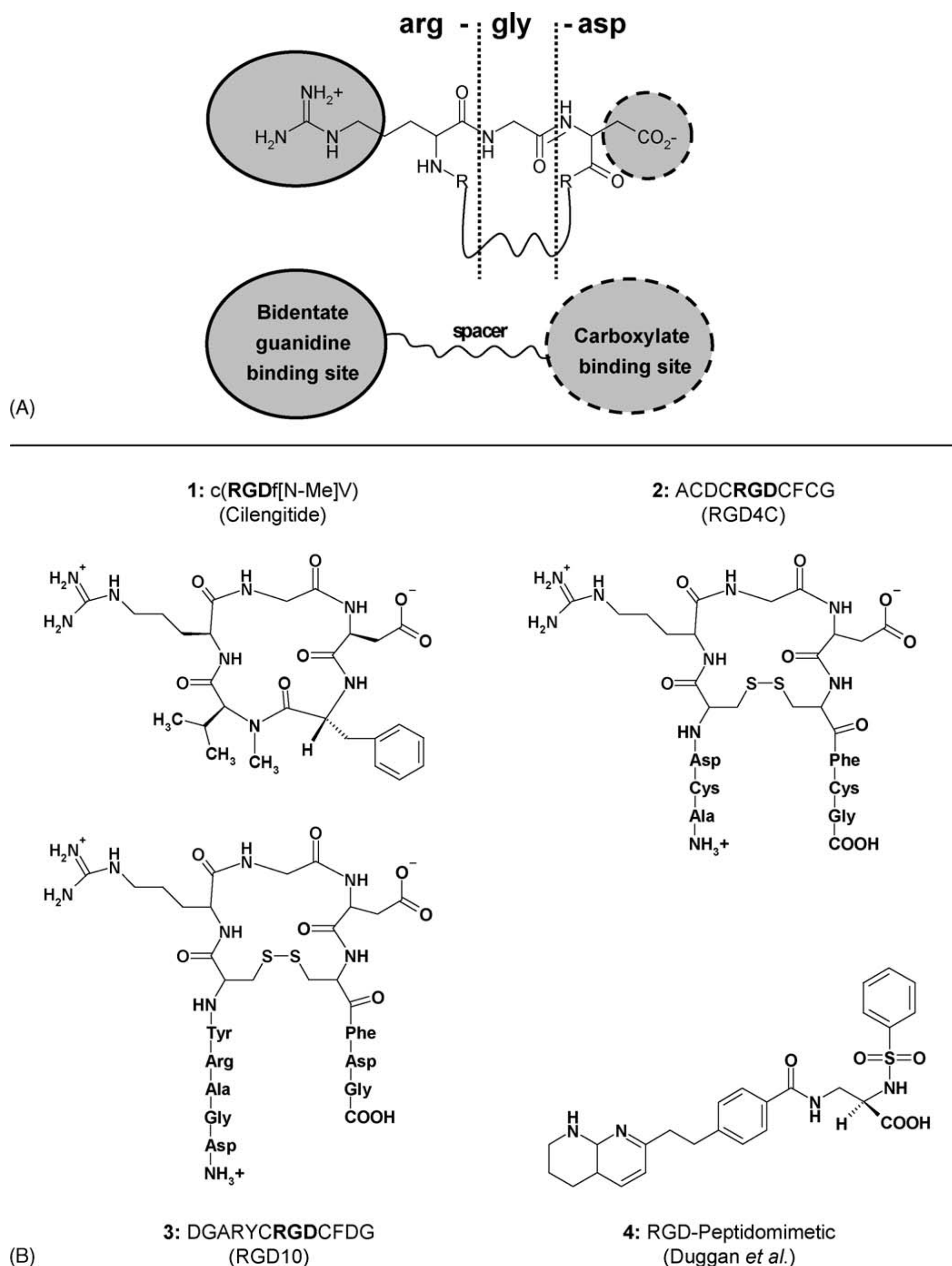

Fig. 2. The original RGD sequence in comparison with modern high affinity ligands for $\alpha_v\beta_3$-integrin. (Panel A) Schematic representation of the RGD binding motif. Specificity and affinity for $\alpha_v\beta_3$-integrin has been introduced in peptide ligands by ring closure and flanking amino acids, which force the arginine and aspartic acid side-chains into the proper conformation. In RGD-mimetics, the two domains that interact with the integrin (Arg and Asp) have been replaced by a guanidine binding site and carboxylate group. (Panel B) Structures of c(RGDf(N-Me)V), RGD4C, RGD10 and RGD-mimetic described by Duggan et al., which are all high affinity ligands for $\alpha_v\beta_3$-integrin.

EMD121974 or Cilengitide (Fig. 2, structure **1**). This RGD-peptide displays affinity in the subnanomolar range in competing with biotinylated vitronectin or fibrinogen for binding to immobilized integrins. Furthermore, Cilengitide displays a 1000-fold preference for $\alpha_v\beta_3$ integrin over $\alpha_{IIb}\beta_3$ integrin (Dechantsreiter et al., 1999). These features were attributed to the constrained ring structure and the introduced D-amino acid residue that force the RGD-sequence into the proper conformation for binding to $\alpha_v\beta_3$ integrin. Cilengitide has reached phase II clinical trials for the treatment of malignancies including melanoma, glioblastoma and prostate cancer. Among the derivatives of this series, c(RGDfK) is one often used for the delivery of therapeutics, because the lysine residue (K) makes it an ideal building block for further chemical conjugation reactions. Several drug targeting and imaging constructs, bearing either one or more c(RGDfK)

ligands, will be highlighted in the following sections of this review.

Another RGD-ligand with high binding affinity and specificity for $\alpha_v\beta_3$ has been developed using phage display. Selection of RGD-peptide ligands from a cyclic peptide library afforded a structure that contained two disulfide bonds (ACDCRGDCFCG) (Fig. 2, structure **2**). This so-called RGD4C peptide was at least 20-fold more potent than similar peptides with a single disulfide bond and 200-fold more potent than commonly used linear peptides (Koivunen et al., 1995). The RGD4C peptide has been exploited as targeting ligand for the delivery of cytostatic drugs; moreover it is especially suitable for incorporation into proteins and viruses by recombinant means. A disadvantage of RGD4C is that the peptide can fold into different cyclic structures. Apart from the preferred bicyclic structure, additional monocyclic and bicyclic structures can form, which demonstrated 10-fold less binding affinity (Assa-Munt et al., 2001).

If one compares c(RGDfK) and RGD4C, c(RGDfK) is preferred for a chemical conjugation approach, due to its higher stability and relative ease of coupling. As mentioned, RGD4C is the best choice to equip a protein with a targeting moiety by recombinant means. RGD peptides with D-amino acids or other chemical modifications cannot be incorporated by this recombinant strategy.

Recently, a novel RGD-peptide with high affinity for $\alpha_v\beta_3$ was discovered by phage display technology (Holig et al., 2004). This peptide, RGD10 (Fig. 2, structure **3**), has only one disulfide bond, but the amino acids flanking the CRGDC core have similar physicochemical properties as the ones in RGD4C. RGD10 and RGD4C displayed similar binding properties.

Several RGD peptidomimetics have been reported with further improved binding to α_v-integrins (Goodman et al., 2002). Most of these compounds contain a guanidine mimetic to replace arginine, while the aspartic acid of the RGD motif has been substituted by a carboxylic acid. These two essential groups have been linked together by various tethers and constraints**,** yielding compounds with low nanomolar or even picomolar affinities for $\alpha_v\beta_3$ integrin (Fig. 2, structure **4**). Only few RGD-peptidomimetics have been exploited for targeting of therapeutics or diagnostics (Hood et al., 2002; Liu et al., 2003; Winter et al., 2003). This is partly due to the fact that they lack groups suitable for the coupling of drug or drug carriers. Likely, peptidomimetics will be applied more often as a targeting moiety, since their excellent binding properties as well as their stability can be regarded ideal for drug delivery.

The affinity as well as the pharmacokinetic properties of RGD-ligands can be improved by coupling them to a carrier system. The multivalent RGD-protein conjugates synthesized by Kok et al. (2002) showed subnanomolar affinity for $\alpha_v\beta_3$ expressing human umbilical cord endothelial cells (HUVEC), which is a 250-fold increase versus the single RGD-peptide. Furthermore, multivalency not only greatly improves affinity but also facilitates internalization (Boturyn et al., 2004; Schraa et al., 2002a). Carrier systems like liposomes, nanoparticles, proteins and other polymers bearing multiple RGD-peptides are therefore more likely to be internalized via receptor-mediated endocytosis than single peptide constructs. Several other common advantages are attributable to RGD-equipped macromolecular carriers even though they represent a diverse group.

1. More drug molecules can be delivered per internalizing receptor/targeted molecule.
2. Higher affinity and internalization are facilitated by multivalent RGD-ligands, as mentioned above.
3. Renal filtration is inhibited since the higher molecular size of the carrier prevents glomerular filtration. This may lead to prolonged blood circulation times and longer presentation of the ligand to target receptors within the tissue (Schraa et al., 2002b).
4. Shielding of drugs from receptors or enzymes in the blood circulation renders targeting more predictable.
5. The high molecular weight of most carriers leads to passive retention in a tumour, via the so-called enhanced permeability and retention (EPR) phenomenon. For example, RGD4C equipped polymers accumulated in a s.c. prostate carcinoma in the course of 3 days, while radioactivity in other organs decreased (Mitra et al., 2005). This resulted in a 50:1 tumour:blood ratio at day 3. The control polymer without RGD-targeting motif accumulated in the tumour to a lesser extent, demonstrating the contribution of RGD-mediated targeting to the EPR effect.

The need for appropriate control to prove the actual role of RGD/integrin recognition has to be mentioned. The substitution of only one amino acid had been demonstrated to abolish interaction with the integrin (Pierschbacher and Ruoslahti, 1987). Most researchers apply RAD or RGE peptides for this purpose. A control construct prepared with such peptides would bear identical structural changes as compared to the RGD modified construct. Comparison in effectiveness of both constructs demonstrates the role of the RGD mediated targeting.

3. RGD-mediated drug targeting approaches

3.1. RGD-mediated delivery of small molecule drugs

The first approach in which the RGD motif was used for drug targeting purposes, rather than as a single antiangiogenic entity, focused on the delivery of doxorubicin to angiogenic endothelial cells. A doxorubicin-RGD4C conjugate (doxo-RGD4C) proved to be equally effective as free doxorubicin in vitro and, more importantly, demonstrated improved inhibition of tumour growth and spreading of metastases in mice (Arap et al., 1998). In addition to an improved efficacy, doxo-RGD4C also displayed reduced toxicity to liver and heart. These promising results were obtained in the MDA-MB-435 breast cancer model, a tumour model in which $\alpha_v\beta_3$

is expressed by the endothelium in the angiogenic blood vessels and by the tumour cells themselves. As such it is difficult to infer whether the effects of doxo-RGD4C were solely due to its vascular effects or results from direct actions on cancer cells as well. A slightly differently synthesized doxo-RGD4C conjugate was tested in a mouse hepatoma model with $\alpha_v\beta_3$-negative MH134 tumour cells (Kim and Lee, 2004). In vitro the conjugate proved to be far less effective than doxorubicin alone, which can be expected in view of the inability of the conjugate to bind to and enter into the MH134 tumour cells. When tested in vivo, the doxo-RGD4C conjugate demonstrated superior antitumour efficacy over free doxorubicin. Since no direct antitumour cell effects of the targeted doxorubicin can be expected in this $\alpha_v\beta_3$-negative tumour model, this result indicates that killing of the angiogenic endothelium by means of an RGD-targeted conjugate can result in a strong antitumour effect.

Several other RGD4C-doxorubicin conjugates have been developed. In order to improve the therapeutic window of the targeted doxorubicin even further, these conjugates were designed as RGD-targeted prodrugs that require activation by tumour-secreted enzymes. Such a strategy would render the cytostatic drug less toxic and more tumour specific, thus allowing far higher doses to be systemically delivered than non-targeted cytostatics. de Groot et al. (2002) developed a prodrug containing a plasmin-specific cleavage site. After accumulation of the prodrug onto $\alpha_v\beta_3$ integrin and subsequent release of the parent drug by plasmin, doxorubicin regained its cytostatic activity. This was exemplified by in vitro studies in which the prodrug was pre-incubated with plasmin, thereby restoring the IC_{50} of the compound to the level of doxorubicin. In addition to doxorubicin-related effects, potential antiangiogenic activity also resides in the RGD4C moiety of this type of compounds, as it can block $\alpha_v\beta_3$ mediated adhesion. The RGD-doxo prodrug was indeed capable of blocking the adhesion of endothelial cells to vitronectin in submicromolar concentrations. However, despite the promising in vitro data, no follow-up studies have been reported in which the plasmin-cleavable prodrug was studied in vivo. Poor water solubility of doxo-RGD4C prodrugs, as well as problems associated with the chemical synthesis of RGD4C-drug conjugates may have obstructed further development of this type of compounds (Burkhart et al., 2004; de Groot et al., 2002; Kim and Lee, 2004). Furthermore, the inherent reversibility of the two disulfide bonds in the peptide may lead to disruption of the bicyclic structure, either during the synthesis of the prodrugs or upon incubation with biological media like serum. Such problems have not been reported for c(RGDfK) based homing ligands, which display a high chemical stability and good water solubility. In a recent comparative study by Burkhart et al. (2004), both types of RGD-peptides were used to target the doxorubicin-derivative doxsaliform. The doxsaliform prodrug is spontaneously converted into an active metabolite of doxorubicin, doxorubicin-formaldehyde, which is more cytotoxic than doxorubicin and also kills doxorubicin resistant cancer cell lines (Cogan et al., 2004; Fenick et al., 1997). By reacting doxsaliform via its prodrug moiety to the RGD-peptides, several $\alpha_v\beta_3$ targeted doxsaliform prodrugs were created. Both types of RGD-prodrugs displayed low nanomolar IC_{50} of MDA-MB-453 tumour cell binding to vitronectin, thus demonstrating their capability to interact with $\alpha_v\beta_3$ integrin. Remarkably, an acyclic RGD4C peptide ligand showed even higher affinity for $\alpha_v\beta_3$ integrin than the bicyclic RGD4C peptide, which is in contrast with earlier reports in which the properly folded structure was regarded as the active structure (Assa-Munt et al., 2001). Since acyclic RGD4C peptide also displayed better water solubility than dicyclic RGD4C, the full conjugate with doxsaliform was prepared with the acyclic peptide. Both this compound as well as the doxsaliform conjugate with c(RGDf[N-Me]V) showed similar pharmacological properties. At physiological pH and temperature, the compounds released doxorubicin-formaldehyde by spontaneous chemical hydrolysis with a half-life of about 1 h, thereby regaining an in vitro IC_{50} about 50–100 nM. Several experiments showed that the RGD-equipped prodrugs did not penetrate the plasma membrane, in contrast to native doxorubicin and the released doxorubicin-formaldehyde. Thus, the proposed mechanism of action of these compounds is initial binding to $\alpha_v\beta_3$ integrin, followed by local release of the more lipophilic doxorubicin species, which can then diffuse through cell membranes.

Besides doxorubicin, several other examples exist in which either the RGD4C peptide or the c(RGDfK) motif was used as an $\alpha_v\beta_3$ integrin targeted ligand. In view of the above-discussed issues on synthesis and solubility problems observed with RGD4C-doxorubicin conjugates, it is understandable that successful synthesis with RGD4C was reported for hydrophilic peptide-like drugs but not with classical cytostatic drugs. The combined synthesis of therapeutic peptides together with RGD4C by solid-phase peptide chemistry avoided most of the concerns raised on the organic synthesis of RGD4C-drug conjugates, while the cationic nature of the final products promoted good water solubility. In contrast, the c(RGDfK) homing motif cannot be incorporated into peptidic constructs with similar ease, since the cyclic peptide needs head-to-tail cyclization to gain its proper binding properties. Indeed, the targeting potential of c(RGDfK) has been demonstrated predominantly in conjugates requiring organic coupling of the RGD-peptide to a drug or drug-carrying macromolecule. Recently, a paclitaxel (PTX) prodrug was reported containing a divalent c(RGDyK) domain, thus promoting better binding affinity than a monovalent conjugate could achieve (Chen et al., 2005). The product (PTX-RGD_2) accumulated in an $\alpha_v\beta_3$-specific manner in MDA-MB-435 tumours (2.7%ID/g at 2 h after injection), which was comparable to the accumulation of the dimeric RGD-peptide without the coupled drug. In order to follow the pharmacokinetics of the PTX-RGD_2 conjugate, the tyrosine residue in the cyclic peptide was iodinated with ^{125}I. This procedure did not affect the binding properties of the peptide and has been applied before to other related cyclic peptides (Haubner et al., 1999).

Since an $\alpha_v\beta_3$-positive tumour was used in these studies, the accumulation of PTX-RGD_2 in the tumour did not reflect targeting to angiogenic blood vessels per se, but a combined homing to tumour and endothelial cells within the tumour tissue. Furthermore, although in vivo antitumour effects were not reported, the PTX-RGD_2 product demonstrated low-nanomolar activity in vitro towards MDA-MB-435 tumour cells. This activity, however, was less compared to that of PTX (IC_{50}: PTX = 34 nM, PTX-RGD_2 = 134 nM). Of note, the conjugate was prepared with a succinate-derivative of PTX, allowing drug release after hydrolysis of the succinate ester. Since no drug release studies were discussed, it is difficult to interpret whether the pharmacological actions of PTX-RGD_2 involved the actual uptake and intracellular processing of the conjugate. Alternatively, PTX may be released outside the cells, either in solution or after binding to $\alpha_v\beta_3$ integrin. We have observed such a premature drug release with one of our products in which the paclitaxel-succinate ester was coupled to a c(RGDfK)-equipped albumin carrier. RGD-targeted PTX-albumin conjugates demonstrated similar HUVEC killing efficacy as non-targeted RAD-PTX-albumin control conjugates (Kok, unpublished results). We therefore concluded that a substantial amount of the drug was hydrolysed from the conjugate before receptor-mediated uptake of the conjugate occurred. Thus, proof of truly targeted intracellular delivery should include demonstration that binding to $\alpha_v\beta_3$ and subsequent internalization is an essential step in the activation of the product. For the PTX-RGD_2 conjugate, this proof could be obtained by competing with free RGD-peptide for the therapeutic activity of PTX-RGD_2 in vitro.

The disappointing results of RGD-PTX-albumin conjugates prompted us to search for alternative linkers displaying better drug-linking properties. We recently developed a novel linking technique for the conjugation of drug to carriers by applying platinum-coordination chemistry. As this so-called universal linker system (ULS) releases the drug via a slow-release profile, a prolonged action of the delivered drug can be expected. We applied this technology to the coupling of the kinase inhibitor PTK787 with albumin in order to inhibit angiogenic processes induced by vascular endothelial growth factor (VEGF). We furthermore equipped the PTK787-albumin conjugates with polyethylenglycol (PEG) moieties as a linker between RGD and albumin, which confer stealth properties to the conjugates. In addition, since PEG shields the drug-modified albumin, reduced immunogenicity and prolonged circulation time can be expected. The RGDPEG-PTK787-albumin demonstrated potent inhibition of VEGF-induced signalling in vitro (Temming et al., manuscript in preparation).

Similar types of RGD-PEG tethers were used to equip liposomes with $\alpha_v\beta_3$-specific homing devices. Around 300 RGD peptides per liposome were reported. These liposomes were furthermore loaded with doxorubicin (Janssen et al., 2003; Schiffelers et al., 2003) or with 5-fluorouracil (5-FU) (Dubey et al., 2004). The levels of accumulation of RGD-targeted liposomes and non-targeted control liposomes in the tumour after i.v. administration to mice with a subcutaneous C26 murine colon carcinoma were similar. However, intravital microscopy demonstrated clustering of RGD-liposomes in tumour blood vessels, while RAD-liposomes and normal PEG-liposomes were diffusely retained in the tumour by EPR. The differences in cellular distribution also explained why doxorubicin-containing RGD-liposomes inhibited the growth of a doxorubicin insensitive C26 carcinoma, while the other control preparations did not show antitumour effects (Schiffelers et al., 2003). Dubey and co-workers showed improved tumour accumulation and superior antimetastatic and antiangiogenic effects of RGD-targeted 5-FU liposomes, when compared to control liposomes. Recently, additional data were provided in which RGD-targeted liposomes were shown to act superior to free doxorubicin (Holig et al., 2004; Xiong et al., 2005a, 2005b). One study applied the novel high-affinity RGD-peptide RGD10, described in Section 2 (Holig et al., 2004). This peptide was modified with a lipid anchor to facilitate straightforward incorporation into the doxorubicin containing liposome. Binding and internalization of these liposomes as well as superior inhibitory effect over free doxorubicin in a C26 tumour model was proven. The various approaches for RGD-mediated targeting of small drug molecules discussed are listed in Table 1.

3.2. RGD-targeting of therapeutic peptides and proteins

As mentioned in the previous section, RGD4C can be appended or inserted into peptidic drugs or proteins by solid phase synthesis or via recombinant techniques. These approaches have been applied both in the preparation of targeted peptides and proteins, as well as in the development of RGD-targeted adenoviruses which may serve as gene-carrying vectors. The present section will discuss various RGD-targeted peptidic constructs, which are also summarized in Table 2, while RGD-targeted viruses are discussed in Section 3.3.

In order to be an appropriate candidate for an RGD-targeted drug targeting approach, drugs must combine potent therapeutic activity in $\alpha_v\beta_3$ expressing endothelial or tumour cells with inadequate pharmacokinetic performance in animal models or patients. These criteria are certainly applicable to the peptide and protein drugs discussed in the current section. More therapeutic proteins are becoming available for the treatment of cancer and other diseases, as evidenced by the increasing number of preclinical and clinical studies on protein type therapeutics. However, poor pharmacokinetics and unacceptable side-effects are often limiting the efficacy of these proteins. For instance, cytokines show potent activity in ill-treatable cancers, but at the same time display a range of other actions within the body, that are not limited to the desired antitumour activity. Cell-specific targeting of a cytokine would greatly improve its selectivity for the target cells within the tumour, thereby increasing the therapeutic window of the compound and at the same time improving

Table 1
RGD-peptide targeted, small molecule drugs

Compound	Drug	Targeting motif	Experimental model	Efficacy compared to non-targeted drug in vitro/in vivo[a]	Reference
doxo-RGD	Doxorubicin	RGD4C	MDA-MB435 mammary carcinoma[b]	=/+	Arap et al. (1998)
	Doxorubicin	RGD4C	MH134 murine hepatoma[c]	−/+	Kim and Lee (2004)
RGD-targeted doxorubicin prodrugs	Plasmin cleavable prodrug	RGD4C	HUVEC	=/n.d.	de Groot et al. (2002)
	Doxsaliform (doxorubicin-formaldehyde)	c(RGDf(N-Me)V)	MDA-MB-435 cells	=/n.d.	Burkhart et al. (2004)
RGD/doxo liposome	Doxorubicin	c(RGDfK)-PEG	C26 murine colon carcinoma[d]	n.d./+	Schiffelers et al. (2003)
	Doxorubicin	RGD10	C26 murine colon carcinoma	n.d./+	Holig et al. (2004)
	Doxorubicin	RGD-PEG	B16	−/+	Xiong et al. (2005a, 2005b)
RGD/doxo nanoparticles	Doxorubicin	c(RGDfC)-PEG	Cl-66 mammary carcinoma[e]	n.d./n.d.	Bibby et al. (2005)
RGD/5-FU liposome	5-Fluorouracil	c(RGDfK)-PEG	B16F10 murine melanoma[b]	/+	Dubey et al. (2004)
PTX-RGD	Paclitaxel	$[c(RGDyK)]_2$	MDA-MB-435 mammary carcinoma[b,e]	−/n.d.	Chen et al. (2005)
RGD/PTX albumin conjugates	Paclitaxel	c(RGDfK)-PEG	HUVEC	=/n.d.	unp.dat.
RGD/PTK787 albumin conjugates	PTK787	c(RGDfK)-PEG	HUVEC	−/n.d.	unp.dat.
RGD/AraC polymers	Arabinoside C	RGDSK	B16 melanoma	−/=	Jasseron et al. (2003)

[a] Improved effect (+), comparable effect (=), less effect (−) or no data (n.d.).
[b] $\alpha_v\beta_3$ integrin positive tumour cells.
[c] $\alpha_v\beta_3$ integrin negative tumour cells.
[d] Doxorubicin insensitive C26 tumour model.
[e] Tumour model only used to assess biodistribution of the compound.

Table 2
RGD-peptide targeted therapeutic proteins and peptides

Compound	Drug	Targeting motif	Experimental model	Efficacy compared to non-targeted drug in vitro/in vivo[a]	Reference
RGD-targeted pro-apoptotic peptides	(KLAKLAK)2	RGD4C	MDA-MB435 mammary carcinoma[b]	+/+	Ellerby et al. (1999)
	Tachyplesin	CRGDC	B16 melanoma	No comparison	Chen et al. (2001)
RGD-endostatin	Endostatin	RGDS	LS174T colon carcinoma	+/+	Yokoyama and Ramakrishnan (2004)
RGD-Fc	Fc fragment of IgG	RGD4C	DU145 prostate carcinoma	n.d./+	Li et al. (2004)
RGD-TNF	hu-TNFα	RGD4C	B16F1 melanoma	n.d./+	Zarovni et al. (2004)
	hu-TNFα mutant selecitve for TNFR1 (V29)	RGDSD	Meth A fibrosarcoma and Mqnu-1 lung cancer	−/+	Kuroda et al. (2000a)
	mTNFα	RGD4C	RMA	=/+	Curnis et al. (2004)
RGD-tTF	Truncated tissue factor	RGD4C	MAD109 and COLON 26	=/+	Hu et al. (2003)
RGD-IL12	mIL12	RGD4C	NXS2 neuroblastoma	−/+	Dickerson et al. (2004)
RGD-antiCD3	Anti-CD3-mAB	c(RGDfK)	HUVEC	+/n.d.	Schraa et al. (2004)

[a] Improved effect (+), comparable effect (=), less effect (−) or no data (n.d.).
[b] $\alpha_v\beta_3$ integrin positive tumour cells.

its efficacy. Besides targeting to surface exposed receptors, either by means of RGD-peptides or via antibody-directed approaches, other structural modifications such as PEGylation may help in improving the pharmacokinetic properties of therapeutic proteins (Mahmood and Green, 2005). Such a strategy would prevent renal clearance, as well as enzymatic degradation of the protein, thus enabling a greater fraction of the administered dose to become available for biological effects.

Similar considerations as discussed above on improving the body distribution and clearance rates are valid for peptidic drugs, which generally have short plasma half lifes. Since (larger) peptides are generally hydrophilic, such molecules depend on specific internalization carriers or mechanisms to access the cytosol. The attachment of an RGD-targeting domain may facilitate peptide drugs' entry into cells. This approach was elegantly illustrated in two studies, which describe the RGD-mediated targeting of cytotoxic peptides. Many cationic peptides display strong disrupting activity towards prokaryotic cells as well as mitochondrial membranes, but are relatively inactive towards eukaryotic cell membranes. Intracellular delivery would allow such peptides to gain access to the mitochondria, eventually leading to target cell apoptosis. This strategy was followed for both a computer-designed pro-apoptotic cationic peptide $(KLAKLAK)_2$, consisting of D-amino acids only, and a naturally occurring antimicrobial cationic peptide, tachyplesin (Chen et al., 2001; Ellerby et al., 1999). Although the CRGDC sequence that was appended to tachyplesin may be less specific for $\alpha_v\beta_3$ integrin, RGD-targeted tachyplesin demonstrated appropriate apoptotic activity in HUVEC and a range of tumour cell lines. Similarly, RGD4C-$(KLAKLAK)_2$ induced apoptosis in Kaposi sarcoma tumour cells. When tested in tumour bearing mice, both products reduced tumour weight and size, while the RGD4C-$(KLAKLAK)_2$ peptide also reduced the formation of lung metastases. In addition, intravenous administration of RGD4C-$(KLAKLAK)_2$ to mice subjected to collagen-induced arthritis reduced the arthritic score significantly (Gerlag et al., 2001). This was attributable to apoptosis of endothelial cells in the inflamed synovium. Hence, targeting to $\alpha_v\beta_3$-positive endothelial cells is not only limited to the treatment of cancer but can also be a feasible therapeutic approach for chronic inflammatory disease, in which angiogenesis is a hallmark of disease progression.

Despite the promising results obtained with RGD-modified pro-apoptotic peptides and with a similar construct in which $(KLAKLAK)_2$ was modified with the CNGRC homing motif (Ellerby et al., 1999), no follow-up studies have been reported on these compounds. Recently, other mechanisms of action by which tachyplesin may exert its activity were reported (Chen et al., 2005). In contrast to the earlier reported mechanism on mitochondrial membrane-disruption eventually triggering apoptosis, it was demonstrated that tachyplesin induced complement activation on the surface of tumour cells. Specificity of tachyplesin for certain cell types, such as TSU prostate tumour cells as well as endothelial cells, was attributed to the hyaluronan-binding site in tachyplesin. Whether RGD-modified tachyplesin has a similar binding preference – for hyaluronan rather than for integrins – or activates complement has not been investigated.

Tumour necrosis factor alpha (TNFα) can exert multiple biological effects on tumours such as apoptosis induction, recruitment of leukocytes, fibrin deposition and vascular leakage (Ferrero et al., 2001). Despite clear beneficial effects of TNFα treatment on tumour progression in animal studies, clinical success in the treatment of cancer is limited due to systemic toxicity. When systemic distribution of TNFα was prevented by local treatment protocols such as isolated limb perfusion, the full potential of TNFα could be exploited (Eggermont et al., 1996; Grunhagen et al., 2004). Thus, it was expected that specific targeting of TNFα to a selective cell population would improve its cytostatic potential.

One of the successfully produced TNFα-variants that was equipped with an RGD homing motif was the TNF mutant V29, in which Arg29 has been replaced by valine. TNF-V29 only binds to TNF-R1, leaving TNF-R2 unaffected (Kuroda et al., 1995). TNF-V29 showed potent antitumour activity in mice, combined with a reduced systemic toxicity (Kuroda et al., 1995; Kuroda et al., 2000a). This observation is in good agreement with data on antivascular activities of TNFα itself which induces vascular permeability via activation of TNF-R1 on endothelial cells (Ferrero et al., 2001). Since the introduction of an RGD-motif in wild-type TNF reduced its gastrointestinal toxicity (Shikama et al., 1994) and improved its antimetastatic activity (Miyata et al., 1995), the RGD-TNF-V29 mutant F4614 was developed to combine both types of recombinant modifications (Shikama et al., 1995). The N-terminal sequence of V29 was altered with the SSS-RGDSDK sequence, which is not specific for angiogenic integrins only. RGD modification imparted fibronectin-like properties in a cell adhesion assay but no quantitative measurement of binding affinity towards $\alpha_v\beta_3$ was made.

Although the in vitro cytotoxic activity of RGD-TNF-V29 on fibroblasts was reduced as compared to wild-type TNFα, the activity on tumour cells and tumour endothelial cells was preserved. Notably, RGD-TNF-V29 displayed considerably lower cytotoxicity in normal endothelial cells, while culturing in tumour-conditioned medium restored the TNF-responsiveness of endothelial cells to the level of wild-type TNFα (Kuroda et al., 2000b). An increased tumour-selectivity of up to 460-fold was reported for RGD-TNF-V29, while wild-type TNF showed only a 4-fold difference in tumour-endothelial selectivity. Tumour conditioned medium probably increased the amount of TNF-R1 on HUVEC, but a possible effect via $\alpha_v\beta_3$-integrin cannot be excluded. When tested in vivo, RGD-TNF-V29 demonstrated a 2-fold improved therapeutic index (LD_{50}/MED = 4.8) (Kuroda et al., 2000a). Part of this improvement might be directly attributed to the improved accumulation in the tumour tissue by virtue of the RGD-modification of the protein, which also prolonged the plasma half-life of RGD-V29 10-fold (Kuroda

et al., 2000a). However, a more pronounced improvement of the therapeutic index had been anticipated on the basis of the observed in vitro selectivity for tumour endothelial cells.

In a different approach, Curnis et al. (2004) prepared a TNFα fusion protein containing the RGD4C sequence at the N-terminus of mouse-TNFα (mTNFα). Since TNFα forms trimers, RGD4C-TNF actually contains three binding domains per protein. Due to this multivalency, RGD4C-TNF showed a 10-fold higher affinity than free RGD4C peptide for α_v-integrins on the endothelial cell line EA.hy926. Further binding experiments revealed that RGD4C-TNF bound to both integrin and TNF-receptor. To demonstrate that RGD4C-modified TNF exerted similar activity as wild-type TNF, the proteins were tested in different in vitro assays either comparing cytotoxic activity or assaying TNF-induced ICAM expression by EA.hy926 cells. In all of these experiments, RGD4C-TNF showed a slightly improved activity profile which was associated with its RGD4C-binding domain, since competition with excess of free peptide could reduce the RGD4C-TNF activity to the level of mTNFα. These results suggest that the binding of RGD4C-TNF to $\alpha_v\beta_3$ integrin improves the pharmacological activity of the protein, apart from merely increasing the number of molecules bound to the surface of the target cells.

The antitumour effect of RGD4C-TNF was evaluated in combination therapy with melphalan in mice bearing s.c. RMA lymphoma tumours. Such an approach was selected since good results had been obtained in isolated limb perfusions with TNFα/melphalan combination therapy (Grunhagen et al., 2004; Hwang and Hunt, 2003). The vascular permeability induced by RGD4C-TNF likely will increase the intratumoural accumulation of the cytostatic drug. Furthermore, previous testing of NGR-TNF, which binds to the vascular protein aminopeptidase N (CD13), had already demonstrated the feasibility of such a vascular targeting strategy for treatment of cancer (Curnis et al., 2000). NGR-TNF displayed a tremendously increased potency versus wild-type TNF, with strong antitumour effects in subnanomolar doses when combined with melphalan. Similarly, only 0.3 ng of RGD4C-TNF showed a strong synergistic antitumour effect with 50 μg of melphalan, while melphalan alone or RGD4C-TNF alone showed no effect, as did the combination of melphalan and wild-type TNFα (also 0.3 ng). In comparison with NGR-TNF, however, the RGD-TNF construct proved less potent since a 3-fold higher dose of RGD-TNF was necessary to achieve comparable effects. Thus, although RGD-TNF proved far more potent than non-targeted TNFα, targeting via CNGRC apparently had delivered more cytokine to the tumour. Most likely, this corresponded to receptor expression levels of $\alpha_v\beta_3$ and CD13 on the tumour vascular wall. NGR-TNF is now in clinical phase I trials, and another targeted cytokine containing the NGR motif, NGR-IFNγ, has recently been reported (Curnis et al., 2005). The RGD-TNF construct, however, may have an added value to NGR-TNF. For instance, receptor expression levels may differ between patients and tumour types, resulting in different relative capacities of the two homing devices. Secondly, the combination of both NGR-TNF and RGD-TNF can amplify the antivascular effect of targeted TNFα since more target receptors will be available for the combined treatment. This may result in more TNF molecules delivered to a single target cell, thus improving its efficacy, or delivery to vessels in different growth stages within the tumour. Since not all vessels express similar levels of either $\alpha_v\beta_3$ or CD13, combination therapy aimed at multiple targets may result in a more widespread antitumour activity of the cytokine.

Similar conclusions on vascular heterogeneity were drawn after studying the effect of three fusion proteins for inducing tumour vessel thrombosis targeted to different epitopes (Hu et al., 2003). Hu et al. compared the efficacy of targeted tTF (truncated Tissue factor), either directed to small, medium sized or larger tumour blood vessels. Among these, RGD4C-targeted tTF was developed to induce blood coagulation at the surface of $\alpha_v\beta_3$ expressing vessel walls. In order to target other antigens on the tumour blood vessels as well, antibody-tTF fusion proteins were constructed with specificity for either fibronectin, which is located in the basal membrane of blood vessels, or for antigens that become expressed during cellular necrosis. In vitro, all fusion proteins retained similar thrombotic activity. After in vivo administration to mice bearing MAD109 lung tumours, RGD-tTF was found by immunohistochemical detection to be localized mainly in capillaries and small vessels of the tumour. Although treatment with daily injections of RGD-tTF ($5\times$ 10 μg of fusion protein i.v.) resulted in thrombosis of about 40% of the tumour blood vessels, this did not result in a significant inhibition of tumour growth. In contrast, the other two fusion proteins displayed thrombosis in up to 80% of the scored blood vessels, leading to massive tumour necrosis and more than 50% reduction in tumour volume versus the RGD-tTF group. Similar treatment results were obtained in the C26 colon carcinoma model. From these results, it was concluded that targeting tTF to angiogenic capillaries and small vessels by means of RGD-tTF caused little damage to the tumour in contrast to targeting bigger blood vessels by the antibody-targeted constructs. Other explanations for the differences in potency reside in the relative affinities of the applied targeting motifs, or the abundance of target receptors within the tumour. Most impressive tumour suppression was observed when all three-fusion proteins were combined. Thus, the delivery of tTF to all available targets produced an additive thrombotic effect, consistent with the observation that different vessels were targeted by each fusion protein.

The RGD targeting approach has also been exploited to redirect an immune response to affect immune-mediated vascular damage or tumour cell killing. Both an immunostimulatory approach was investigated in which the cytokine IL-12 was modified with RGD-motifs, and an approach in which cytotoxic T-cells were redirected to endothelial cells. For this latter approach, anti-CD3 was modified with up to 25 c(RGDfK) groups (Schraa et al., 2004). The resulting RGD-anti-CD3 conjugate showed high affinity for

$\alpha_v\beta_3$ and maintained its capacity to bind to cytotoxic T-lymphocytes. In vitro, the conjugate induced CTL-mediated lysis of HUVEC cells in an RGD-dependent manner. Whether such an approach can be successful in vivo needs to be evaluated.

IL-12 is recognized for its immunostimulatory properties, such as activation of natural killer (NK) cell activity and induction of IFNγ production by NK cells and T cells. Systemic administration of IL-12 was, however, associated with dose-limiting toxicity, thus preventing IL-12 from attaining its full therapeutic potential (Car et al., 1999). Hence, a fusion protein was synthesized linking mouse IL-12 to RGD4C (Dickerson et al., 2004). RGD-IL-12 retained the immunostimulatory activity of IL-12, although moderately higher doses were needed to effectuate in vitro production of IFNγ by mouse splenocytes. An unexpected increased efficacy was observed when the compound was tested in a corneal angiogenesis assay. An almost complete inhibition of bFGF-induced vessel growth was observed when mice were treated with RGD-IL-12, while native mIL-12 only partially inhibited neovascularization. Similarly, RGD-IL-12 showed an improved antitumour effect when tested in a neuroblastoma model (NXS2 model), while native IL-12 was not effective. Furthermore, RGD4C-mediated targeting prevented IL-12 induced hepatic necrosis, which was observed after continuous s.c. infusion for 2 weeks via surgically implanted osmotic pumps. Vascular targeting of the cytokine thus improved the therapeutic window of the compound by both affecting the tolerability and the efficacy. The improved effect of RGD-IL-12 was due to an improved delivery of IL-12 to angiogenic vessels, but part of the effect also resulted from an IL-12 independent mechanism, likely depending on the RGD4C moiety of the targeted cytokine. When RGD-IL-12 was tested in knockout mice lacking the IL-12 receptor, RGD-IL-12 still was capable of inhibiting neovascularization for up to 25%, while mIL-12 was completely ineffective. Since the coupling of RGD4C to IL-12 would prolong the half life of the peptide and alter its body distribution, such an effect could be observed with RGD-IL-12 but not with an equivalent dose of the free RGD4C peptide. Thus, it is likely that this 25% inhibition is accounted for by RGD4C.

3.3. RGD-peptide mediated delivery of therapeutic nucleic acids

Since DNA and other types of nucleic acids cannot cross the cellular membrane due to their high molecular weight and strong negative charge, such therapeutics require delivery vehicles to reach their intracellular site of action. Although viruses have been unsurpassed in their ability to deliver DNA into cells, concerns exist about their safety. As an alternative approach, non-viral delivery systems for nucleic acids have been developed (Mastrobattista et al., 2005). Complexation of DNA with cationic polymers, peptides or lipids is a widely used technique for non-viral gene delivery. In principle, cation-complexed DNA can interact with the negatively charged proteoglycans on the cell membrane, eventually leading to uptake of the genetic material. Although this non-selective approach is generally applicable to in vitro transfection of cells, targeting ligands need to be introduced in the complex for in vivo delivery to specific cell types. As RGD-targeted systems are internalized after binding, RGD-modification of non-viral and viral gene carriers has successfully been exploited.

3.3.1. RGD-targeted non-viral gene carriers

3.3.1.1. Cationic polymers. The cationic polymer polyethylenimine (PEI) has been investigated by several groups for RGD-guided transfection of angiogenic endothelial cells and tumour cells. While initial studies mainly reported in vitro transfection, more recent studies also demonstrated in vivo effectiveness of RGD-assisted gene delivery. As a general approach, RGD-equipped DNA complexes were prepared by covalent conjugation of the RGD-peptide to the polymer, after which the now RGD-modified PEI was used to complex DNA plasmids or short interfering RNA (siRNA). Different synthesis protocols yielded polymers in which the peptide had been coupled directly to the polymer backbone (RGD-PEI) (Erbacher et al., 1999; Kunath et al., 2003), or polymers in which the peptide had been coupled to the distal end of PEG tethers (RGD-PEG-PEI) (Kim et al., 2004; Kunath et al., 2003; Schiffelers et al., 2004; Suh et al., 2002; Woodle et al., 2001). An important property of the resulting DNA/polymer complexes is the net surface charge, which is defined by the ratio of DNA and polymer in the particles. A high (positive) surface charge may lead to unwanted interactions with non-target cell types, a problem that is circumvented by PEGylation, since these groups shield the charged polymer/DNA complex. This explains why a more pronounced targeting effect was reported for RGD-PEG-PEI, while RGD-PEI showed similar gene transfection compared to conventional PEI/DNA complexes (Erbacher et al., 1999; Kunath et al., 2003; Suh et al., 2002). As not all authors reported successful targeting, parameters like RGD density and PEG density seem important for facilitating receptor clustering and uptake of the particles.

RGD-PEG-PEI was used to deliver siRNA against VEGF and VEGF receptors to angiogenic vasculature in neuroblastoma N2A tumour-bearing mice (Schiffelers et al., 2004). Repeated injections every 3 days resulted in a strong inhibition of tumour angiogenesis and tumour growth, together with a marked loss of peritumoural vascularization. In a similar approach, siRNA delivery was applied to inhibit herpes simplex infection induced angiogenesis in the eye (Kim et al., 2004). siRNAs targeting either VEGF, VEGFR1, VEGFR2 or a mix of the three were used. Local as well as systemic administration of RGD-PEG-PEI containing siRNAs significantly inhibited neovascularization, and mixtures of three siRNAs surpassed the effects of each of the siRNAs alone. In the same study, RGD-PEG-PEI was also employed for the gene delivery of soluble VEGFR1 into angiogenic endothelial cells (Kim et al., 2004). After in vitro transfection of endothelial

cells, the soluble receptor produced by the cells inhibited VEGF-induced endothelial cell proliferation by scavenging the growth factor.

Although cationic polymers have the advantage of relatively simple synthesis methods for introduction of different structures, one of the concerns of these polymer-based materials is their lack of biocompatibility and biodegradability. Cationic constructs based on lipids and peptides have been explored to overcome this disadvantage.

3.3.1.2. Cationic lipids. Hood et al. (2002) used RGD-modified cationic lipid-based nanoparticles to deliver a mutated form of Raf-1 to angiogenic blood vessels. Raf plays an important role in angiogenic intracellular signalling pathways, and blockade of Raf activity has been shown to suppress angiogenesis (Eliceiri et al., 1998). To deliver the plasmid encoding the mutated Raf to angiogenic endothelial cells, the lipid particles were equipped with an $\alpha_v\beta_3$ selective RGD-mimetic (Duggan et al., 2000). The resulting particles showed in vitro selectivity for $\alpha_v\beta_3$ expressing M21 tumour cells. When mice bearing M21-L tumours (the $\alpha_v\beta_3$-negative variant of the M21 melanoma) were treated with a single intravenous dose of these nanoparticles, apoptosis of tumour endothelium was observed, as well as apoptosis among the tumour cells proximal to apoptotic vessels. The absence of $\alpha_v\beta_3$ on the tumour cells suggests that the latter effect was due to the vascular effect, rather than a direct effect on the tumour cells. In a therapeutic setting, the RGD-targeted gene encoding for the Raf-mutant achieved tumour regression in the majority of animals, an effect reported to last for more than 250 days. Furthermore, when tested in the $\alpha_v\beta_3$-negative CT26 colon carcinoma model, RGD-targeted nanoparticles reduced the number of lung and liver metastases, an effect not observed for the non-targeted particles.

Several other studies report on RGD-equipped DNA lipoplexes (Table 3). As has been described for polymeric carrier systems, particles prepared with cationic lipids required shielding of positively charged groups for proper gene delivery in vivo.

3.3.1.3. Cationic peptides. Peptides with cationic lysine residues have been popular complexing agents for nucleic acids. In combination with the buffering capacity of histidines, efficient carrier systems can be developed. Aoki used a linear peptide vector of 36 amino acids with the sequence CRGDCF(K[H-]KKK)6. In vitro, luciferase expression in hepatoma and pancreatic cancer cell lines could be abolished in the presence of an excess of c(RGDfV), demonstrating that RGD/integrin interaction was involved in the intracellular delivery of the reporter gene. In vivo, luciferase activity in tumour tissue was significantly higher than expression in lung, kidney, and spleen, and slightly higher than expression in the liver. The developed RGD-targeted DNA complexes were applied for the delivery of siRNA and antisense DNA against c-Raf. Both approaches successfully reduced c-Raf levels, with siRNA being far more potent than antisense.

A branched version of lysine-histidine peptides with a terminal RGD-targeting peptide was developed in the group of Mixson. Their work illustrates the strong dependence of transfection efficiency on the branching pattern and sequence of the HK-peptides. Both delivery of reporter genes and silencing of reporter genes by siRNA occurred more efficiently for RGD-HK peptides as compared to non-targeted DNA lipoplexes (Leng et al., 2005; Leng and Mixson, 2005).

3.3.1.4. Mixed systems. Various combinations of cationic polymers, lipids and peptides have been reported for gene delivery. As these studies mainly report in vitro transfection of tumour and endothelial cells, we will not discuss them in detail. Only approaches that apply carrier systems different from the ones already discussed are addressed below.

Since the polysaccharide schizophyllan (a natural β-(1-3)-D-glucan) contains a domain that can complex with homopolynucleotides, this carrier can be used for non-viral gene delivery. For this purpose, antisense against the transcription factor *c-Myb* was modified with a homopolynucleotide tail (polyA_{40}) and RGD moieties were coupled to the schizophyllan carrier. These constructs were capable of inhibiting the proliferation of A375 cells by approximately 80% (Matsumoto et al., 2004).

A proteinaceous gene carrier was engineered by equipping β-galactosidase with an oligolysine tail and an RGD-targeting motif (Aris and Villaverde, 2000). Using recombinant expression techniques, the RGD containing segment of the capsid protein VP1 of foot-and-mouth-disease virus was introduced into β-galactosidase. The addition of a $(Lys)_{10}$ (deca-lysine) tail to the amino terminus was used to complex DNA. The construct was shown to bind and transfect HeLa cells.

PEI-complexed DNA was coated with anionic phospholipids conjugated to cyclic RGD-peptides to resemble the structure of viral particles (Fahr et al., 2002; Muller et al., 2001; Nahde et al., 2001). The transfection level reported for these artificial virus particles in endothelial cells and melanoma cells correlated to the level of $\alpha_v\beta_3$-integrin expression. To enhance the specificity of the vector for tumour cells, the RGD-equipped vector was combined with transcriptional targeting using a melanoma-specific tyrosinase-based promoter system, which increased specificity for melanoma approximately 500-fold.

3.3.2. RGD-mediated viral gene delivery

At present, viruses remain the most efficient vectors for introducing genetic material into mammalian cells. A variety of viruses display RGD-containing proteins on their surface through which they bind to integrins (Bai et al., 1994; Chu and Ng, 2004; Isberg and Tran Van, 1994; Roivainen et al., 1994; Vene et al., 2000). As the initial cell adherence of adenoviruses is mediated via other proteins, like binding of the capsid protein fibre to the Coxsackie adenovirus receptor (CAR), RGD-integrin interactions are thought to mediate internalization (Albinsson and Kidd, 1999; Hong

Table 3
RGD-mediated gene delivery using non-viral envelopes

Construct	Gene	Targeting motif	Experimental model	Efficacy compared to non-targeted vector in vitro/in vivo[a]	Reference
Polymers					
RGD-PEI	Luciferase	CYGGRGDTP	HeLa, MRC5 cells	+/n.d.	Erbacher et al. (1999)
	Luciferase	RGDC	Mewo, A549 cells	+/n.d.	Kunath et al. (2003)
RGD-PEG-PEI	Luciferase	RGDC	Mewo, A549 cells	−/n.d.	Kunath et al. (2003)
	siVEGF, siVEGFR1, siVEGFR2	RGD10	Inflammatory angiongenesis in the eye	n.d./+	Kim et al. (2004)
	Luciferase, siVEGFR2	RGD10	Neuro 2A neuroblastoma	+/+	Schiffelers et al. (2004)
	Soluble Flt-1	RGD4C	CDAM endothelial cells	+/n.d.	Kim et al. (2005)
Lipids					
Polymerized lipid nanoparticle	ATPμ-Raf	RGD mimetic from Duggan et al.	M21-L[b]/CT26 colon carcinoma[b]	+/+	Hood et al. (2002)
Cationic liposome	Luciferase	CDMRGDMFC	HUVEC	+/n.d.	Anwer et al. (2004)
Neutralized cationic liposome	CAT	RGD4C	PO2 colon carcinoma	n.d./+	Thompson et al. (2005)
Peptides					
Pronectin F+	LacZ	Fibronectin 17aa	Meth-AR-1 fibrosarcoma	n.d./=	Hosseinkhani and Tabata (2004)
PEG-Pronectin F+	LacZ	Fibronectin 17aa	Meth-AR-1 fibrosarcoma	n.d./+	Hosseinkhani and Tabata (2004)
RGD-HK-linear peptides	Luciferase	CRGDC	Hs700T pancreatic carcinoma	+/+	Aoki et al. (2001)
	AS-c-Raf, si-c-Raf	CRGDC	Hepatoma and pancreatic cell lines	+/n.d.	Aoki et al. (2003)
RGD-HK-branched peptides	siLacZ, siLuciferase	RGD	MDA-MB-435[c], MCF7, SVR-bag4	+/n.d.	Leng et al. (2005)
Mixed systems					
RGD-PEG-lipid protamine-DNA	Luciferase	RGD4C	MDA-MB-435[c], Huh7[b] cells	+/n.d.	Harvie et al. (2003)
RGD-schizophyllan	polyA-AS-c-myb	CRGD	A375 and HL-69 cells	+/n.d.	Matsumoto et al. (2004)
RGD-rec-β-galactosidase-K_{10}	Luciferase	FMDV VP1 capsid 27 aa	Caco-2 and HeLa	+/n.d.	Aris and Villaverde (2000)
Artificial virus-like particle	Luciferase	RGD4C	HUVEC and a range of different tumour cell lines	+/n.d.	Fahr et al. (2002), Muller et al. (2001), Nahde et al. (2001)

[a] Improved effect (+), comparable effect (=), less effect (−) or no data (n.d.).
[b] $\alpha_v\beta_3$ integrin negative tumour cells.
[c] $\alpha_v\beta_3$ integrin positive tumour cells.

and Boulanger, 1995; Mizuguchi et al., 2002). Deletion of the RGD sequence impaired internalization, while addition of flanking amino acids resulted in increased affinity for $\alpha_v\beta_3$-integrin (Albinsson and Kidd, 1999; Wickham et al., 1997).

Several studies report on the introduction of additional RGD-peptides into adenoviruses to redirect them to tumour cells or angiogenic blood vessels (Table 4). As introduction of new RGD-motifs in the fibre would result in surface exposure on the viral capsid, this allows facile recognition by $\alpha_v\beta_3$-integrin. RGD-modified adenovirus (Ad.RGD) showed increased gene delivery to endothelial cells and smooth muscle cells that are normally not transduced by adenovirus due to low expression levels of CAR (Wickham et al., 1997). Later studies demonstrated the feasibility of Ad.RGD gene delivery systems in transfecting malignant cells and HUVEC (Dirven et al., 2002; Kasono et al., 1999; Vanderkwaak et al., 1999).

As an alternative to RGD modification, viral fibre protein was modified with a $(lysine)_7$ (pK_7) motif. The pK_7 motif has been suggested to bind to the heparan sulphate receptor, which is present on a high number of cell types (Fromm et al., 1995). However, the strong positive charge of the clustered lysines may also induce transfection via non-specific cell interactions. Both RGD and pK_7 viruses have been tested in vitro on smooth muscle cells. Although expression of β-galactosidase in smooth muscle cells was improved 10-fold for the RGD-modified virus as compared to control virus, the pK_7 variant induced a 35-fold increase in transgene expression. When both modifications were combined, further increased viral infectivity was reported (Wu et al., 2004).

Table 4
Gene delivery using RGD-modified viruses

Construct	Gene	Targeting motif	Experimental model	Efficacy compared to non-targeted virus in vitro/in vivo[a]	Reference
Ad.RGD	LacZ	RGD4C	A549, 293, CPAE, HISM, A-10, B16-F1 and more cell lines	+/n.d.	Wickham et al. (1997)
	Luciferase	RGD4C	B16 melanoma	n.d./+	Koizumi et al. (2003)
	Luciferase with flt-1 promotor	RGD4C	Endothelial cells, aortae	+/n.d.	Work et al. (2004)
	IL-12, TNFα	RGD4C	B16 melanoma	n.d./+	Okada et al. (2004)
	Thymidine kinase	RGD4C	SKOV3.ip1, OvCAR, Ascites, primary ovarian cancer cells	+/n.d.	Hemminki et al. (2001)
	Thymidine kinase with COX-2 promotor	RGD4C	Oz, SkChA-1 cells	+/n.d.	Nagi et al. (2003)
	Thymidine kinase	RGD4C	B16 melanoma	n.d./+	Mizuguchi and Hayakawa (2002)
Ad.pK7.RGD	Luciferase	RGD4C	SKOV3.ip1 ovarian carcinoma	+/=	Wu et al. (2004)
	Luciferase	RGD4C	HeLa cervical carcinoma	n.d./+	Rein et al. (2004)
	Luciferase	RGD4C	B16 melanoma	n.d./+	Koizumi et al. (2003)
RGD-targeted CRAd	CRAd.Δ24	RGD4C	SKOV3.ip1 ovarian carcinoma	n.d./+	Bauerschmitz et al. (2002)
	CRAd.Δ24	RGD4C	IGRG121 primary glioblastoma	n.d./+	Lamfers et al. (2002)
	CRAd.Δ24	RGD4C	U-87MG glioma[b]	n.d./+	Fueyo et al. (2003)
	CRAd.Δ24	RGD4C	OS-1a primary osteosarcoma	n.d./+	Witlox et al. (2004)
	CRAd.COX-2	RGD4C	SKOV3.ip1 ovarian carcinoma	=/+	Kanerva et al. (2004)
	CRAd.COX-2	RGD4C	Oe19 oesophageal carcinoma	n.d./=	Davydova et al. (2004)
	CRAd.tyrosine enhancer	RGD4C	SK-Mel-28, YUSAC2 melanoma	n.d./+	Liu et al. (2004)
	CRAd.survivin	RGD4C	MDA-MB-361 mammary carcinoma	n.d./+	Zhu et al. (2005)
Avoiding natural tropism	Luciferase	RGD4C	Various cancer cell lines	+/n.d.	Borovjagin et al. (2005)
	Luciferase	PEG-c(RGDfK)	HUVEC	+/n.d.	Ogawara et al. (2004)

[a] Improved effect (+), comparable effect (=), less effect (−) or no data (n.d.).
[b] $\alpha_v\beta_3$ integrin positive tumour cells.

When administered in vivo, Ad.RGD and RGD.pK$_7$ adenoviruses displayed a markedly different gene delivery profile as compared to unmodified viruses. After intravenous administration, RGD-directed viruses caused higher gene expression of reporter gene in liver, lungs, spleen and kidneys. Kidney expression was most markedly changed reaching 88-fold higher levels of expression. Expression in the heart was reduced for RGD-targeted adenovirus as compared to the native form (Reynolds et al., 1999). However, the intravenous route is not very often employed for adenoviral gene delivery. Since RGD-modification does not prevent uptake of the particles via CAR, the majority of the viral load is cleared from the circulation via normal viral tropism, thus preventing adequate viral titres in the tumour. Most studies therefore report intratumoural injection of the constructs.

Although the problems observed after systemic administration of viral gene carriers can be evaded by intratumoural injection, strategies that prevent uptake by the mononuclear phagocyte system require further development. A recent approach to abolish CAR interaction is the chemical modification of the viral surface with PEG-groups, thus affording stealth properties as previously reported for liposomes and other drug carriers (Ogawara et al., 2004). Coupling of PEG to the virus abrogated CAR-knob interaction for both HeLa-cells and HUVEC, showing the effective shielding by the PEG-molecules. Subsequent coupling of c(RGDfK) to the terminal ends of the PEG-chains allowed retargeting of the Ad.PEG-RGD virus to cells expressing $\alpha_v\beta_3$-integrin. Ad.PEG-RGD was able to transduce endothelial cells in vitro. Although the RGD-PEG equipped viral carrier was not evaluated in vivo, similar vectors prepared with anti-E-selectin antibody as a targeting device demonstrated an improved stealth character when administered to mice (Ogawara et al., 2004).

The Ad.RGD and Ad.RGD.pK$_7$ vector have been evaluated in ovarian and cervical carcinoma and in melanoma. In general, Ad.RGD.pK$_7$ showed best infectivity, although this did not apply to all the studied models (Koizumi et al., 2003; Rein et al., 2004). Taken together, these data indicate that infectivity-enhanced vectors may each exhibit distinct values that are dependent upon tumour origin, administration route, and tumour microenvironment.

RGD-modified adenoviruses have been successfully employed for the intratumoural delivery of the inflammatory cytokines IL-12 and TNFα in mice bearing B16 melanoma (Okada et al., 2004). Intratumoural injection of Ad.RGD

provided highest levels of luciferase expression in the tumour while expression levels in liver and spleen were approximately three to 5-fold lower, indicating that spreading to the systemic circulation occurred. The resulting high doses of TNFα produced by the cells transfected with the adenovirus led to deleterious side effects featured by pronounced body weight loss or even death of the animals. Nevertheless, intratumoural injection of safe doses led to inhibition of tumour growth for both cytokines, and the effects were more pronounced when both cytokines were combined.

The specificity of Ad.RGD-mediated transgene expression for angiogenic endothelial cells was further improved by combination with the endothelial specific promoter flt-1 (Work et al., 2004). A 15-fold increase in transfection selectivity was observed for the viruses equipped with the flt-1 promoter, both in cultured cells and in ex vivo transfected aortae. Although, Ad.flt-1.RGD transfection was not evaluated in tumour models, this approach may be applicable to tumour angiogenesis as well.

Viral gene delivery can be applied for the transfection of target cells with enzymes that can activate prodrugs via the so-called Virus Delivered Enzyme Prodrug Therapy (VDEPT) strategy. A commonly applied enzyme for this purpose is the herpes simplex virus thymidine kinase (HSVtk), which can activate antiviral compounds such as ganciclovir. As the locally produced drug can escape from transfected cells and affect bystander cells, VDEPT approaches may exert effects on both $\alpha_v\beta_3$ positive cells and neighbouring cells that are not displaying the integrin. RGD-mediated targeting of the HSVtk virus was evaluated in ovarian carcinoma cells (Hemminki et al., 2001). On average, approximately 20-fold increased levels of thymidine kinase mRNA were detected after infection with Ad.RGD compared to infection with control virus. Importantly, Ad.RGD retained most of its cell killing effect in the presence of ascites. This showed the ability of the mutant virus to escape from neutralizing antibodies that may be present in the ascitic fluid.

The VDEPT strategy with RGD-targeted HSVtk-expressing vectors was further evaluated in B16 melanoma-bearing mice (Mizuguchi and Hayakawa, 2002). Following intratumoural injection of the Ad.RGD-tk virus, ganciclovir was injected intraperitoneally for 10 days. The RGD-targeted virus showed approximately 25 times more antitumour activity than Ad-tk, which furthermore coincided with less liver damage due to spill-over of the constructs to the circulation.

An additional level of specificity was added to the HSVtk virus by placing the thymidine kinase expression under the control of a tumour-specific promoter system (Nagi et al., 2003). After comparison of several promoter strengths, the COX-2 promoter was selected since it showed highest activity in cholangiocarcinoma. Combination of the COX-2 driven promoter with Ad.RGD increased the transgene expression of reporter genes by approximately two orders of magnitude as compared to CMV-driven expression, and about 2–3-fold as compared to non-targeted COX-2.Ad. Thus, apart from increasing the tumour-specificity of virus by using the COX-2 promoter, transgene expression was also enhanced in this specific tumour cell type. The enhanced expression of thymidine kinase coincided with enhanced cytotoxicity in the presence of ganciclovir.

3.3.2.1. Conditionally replicating adenoviruses. Although adenoviruses are considered the most efficient system for gene delivery, in vivo transduction of tumour masses is still largely inefficient and reaches only a portion of the tumour cells. Administration of higher doses would not solve this problem, as this leads to side-effects due to transfection of non-tumour cells, especially in the liver. To overcome this limitation, replicating adenoviruses have been developed which upon lysis of the transfected cancer cells release new virions. Since such replicating viruses are far more toxic than non-replicating viruses, confinement of their action to tumour cells by tumour-specific transcription is a prerequisite. Several activation pathways have been investigated for these so-called conditionally replicating adenoviruses (CRAds), and CRAds have been combined with RGD-assisted transfection.

The mutated retinoblastoma pathway (Δ24), frequently encountered in malignant cells, is one of the most popular approaches to achieve replication of CRAd in malignant cells. Bauerschmitz et al. used the CRAd.Δ24.RGD for treating ovarian carcinoma. Oncolysis of ovarian carcinoma cell lines in vitro was similar to that of replicating Ad.RGD not equipped with the tumour-specific promoter. In a mouse model of peritoneally disseminated ovarian cancer, three doses of 5×10^8 viral particles promoted survival of mice for over 90 days, whereas all control animals had died by day 60 (Bauerschmitz et al., 2002).

This approach has been expanded to other cancer types. Glioma cells, also frequently showing low CAR expression and possessing a mutated retinoblastoma pathway, were more efficiently infected by RGD-modified CRAd than by particles lacking the RGD-peptide. Treatment with CRAd.Δ24.RGD of s.c. glioblastoma-bearing mice resulted in dose-dependent therapeutic effects (Lamfers et al., 2002). Interestingly, combination of viral treatment and conventional irradiation allowed the use of a 10-fold lower viral dose for achieving survival in all treated mice. In an orthotopic glioma model, treatment with CRAd.Δ24.RGD caused survival over 140 days of the majority of animals (Fueyo et al., 2003). Histological analyses of brain tissue at the end of the study revealed complete tumour regression, absence of signs of inflammation, and absence of viral proteins. Similar results were reported for osteosarcoma (Witlox et al., 2004).

The COX-2 promoter, which is frequently upregulated in ovarian cancer cells, has been explored as an alternative system for conditional replication. In vitro, CRAd.COX-2.RGD displayed oncolysis to the same extent as wildtype virus but approximately 100-fold less than CRAd.Δ24.RGD. Both RGD-targeted CRAds were equally efficient in prolonging survival time in an orthotopic mouse model of ovarian cancer, indicating that in vitro oncolytic potency is just one of

the factors that should be considered when optimizing CRAd (Kanerva et al., 2004). The CRAd.COX-2.RGD vector has also been tested in oesophageal adenocarcinoma (Davydova et al., 2004) and bladder carcinoma (Tekant et al., 2005). Although a modest increase was observed in viral transfection of tumour cells with CRAd.COX-2.RGD as compared to CRAd.COX-2 without RGD modification, no differences were observed on tumour inhibition in vivo. In contrast, Ad5/Ad3.CRAd.COX-2 in which the Ad5 knob was replaced by an Ad3 knob, an alternative to RGD for changing the viral tropism, demonstrated a 50–60-fold improved transfection activity. As the Ad5/Ad3 modified CRAd also displayed most promising tumour inhibition in vivo, RGD-CRAd vectors were abandoned in favour of the Ad5/Ad3 vector.

Despite the sometimes spectacular preclinical data, CRAd monotherapy has produced only modest therapeutic effects so far (Ganly et al., 2000; Kirn, 2001; Nemunaitis et al., 2001). It has been suggested that incorporation of additional cytotoxic genes in CRAds may enhance their antitumour effects. However, this would involve the insertion of additional genetic information into the viral genome, which usually requires alteration of the adenoviral E3 region. E3 encodes the adenoviral death protein and loss of this protein significantly impaired the oncolytic properties of replicating adenoviruses (Suzuki et al., 2002). The introduction of additional genetic material should therefore be carried out while conserving the E3 region.

The studies discussed and others included in Table 4 collectively show a multitude of promoter systems that may confer cell-type specific replication. In addition, several strategies have been identified for enhancement of the infectivity of viruses, leading to a large number of possible combinations for CRAd design. A recent study demonstrated a strong variation in CRAd replication for different constructs – wild-type, RGD and Ad5/Ad3 targeted systems in combination with COX-2, Δ24 promoters – between different primary ovarian carcinoma spheroids, indicating that individual selection of CRAds may be an important prerequisite for clinical therapeutic success (Lam et al., 2004).

3.4. RGD-equipped imaging agents

RGD-peptides have also been applied for imaging of tumours and angiogenesis. Sensitive imaging techniques for angiogenesis allow detection of tumour metastases prior to the start of drug treatment, and can be employed to monitor the therapeutic effect of antiangiogenic therapy. Besides detecting $\alpha_v\beta_3$ expression within certain tumours, and thereby providing grounds for an RGD-based drug delivery strategy, RGD-based imaging methods will assist in monitoring drug delivery pharmacotherapy. RGD-based strategies for angiogenesis imaging have recently been reviewed by Haubner et al. (2003). We will therefore only briefly address this topic, focusing on some recent approaches.

During the past years, different types of imaging constructs directed to α_v-integrins have been developed (Table 5). Apart from RGD-peptides, the anti-α_v antibody LM609 has been evaluated for detection of blood vessel formation. LM609-targeted contrast agents successfully detected angiogenesis in cancer as well as in other diseases (Anderson et al., 2000; Sipkins et al., 1998). Although RGD-peptide ligands cannot compete with the high specificity of a monoclonal antibody, the affinity of the peptide can be increased by conjugation of multiple ligands to a backbone or a particle, similar as discussed for drug delivery purposes. Furthermore, the chemical versatility of RGD-peptides and peptidomimetics allow for conjugation to various labels such as ^{125}I, ^{18}F, and chelating agents for metal nuclides like ^{99}Tc, ^{111}In. Another advantage of RGD-peptides is their crossover application in different species. Thus, one may test the same construct in different animal models and in man without the need to redesign or humanize the molecule.

For both drug delivery constructs and for tracer molecules, $\alpha_v\beta_3$ integrin can serve as a target receptor that is expressed by angiogenic endothelial cells, and tumour cells, depending on the type of tumour studied. So far, imaging of angiogenesis has mainly been successful in $\alpha_v\beta_3$-positive tumours by virtue of the higher amounts of tracer that accumulate in the tumour (Haubner et al., 2001b, 2004). More recent studies, however, deal with models in which $\alpha_v\beta_3$ is only expressed on angiogenic vessels, either in cancer or other diseases such as inflammation-associated angiogenesis (Lee et al., 2005; Lewis, 2005; Pichler et al., 2005).

The majority of RGD-peptide-based imaging constructs have been developed based on the c(RGDfV) template, which was modified to optimize the pharmacokinetic properties of the tracer for imaging. In general, low hepatic clearance and moderate renal elimination are preferred for the tracer, since this will yield a molecule, which can achieve high tumour/blood ratios without image-interfering liver uptake. Since initial ^{125}I-iodinated peptides such as c(RGDyV) showed mainly hepatic clearance, more hydrophilic compounds were pursued, for instance by replacing part of the cyclic structure with a sugar-moiety (Haubner et al., 2001a). The resulting sugar-amino acid compound also called ^{125}I-Gluco-RGD maintained its specificity for $\alpha_v\beta_3$, while reduced liver uptake was demonstrated (Haubner et al., 2001a). Further optimization of the tracer led to the ^{18}F-Galacto-RGD ligand, a tracer suitable for positron emission tomography (PET) (Haubner et al., 2001b). In parallel, several other hydrophilic c(RGDfV)-based tracers have been reported, in which the RGD-peptide was either modified by appending more hydrophilic labelling reagent such as 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) or diethylenetriaminepentaacetic acid (DTPA), or by coupling of hydrophilic tethers such as hydrophilic amino acids or PEG-chains (Chen et al., 2004a, 2004d; Haubner et al., 2003). Since PEGylation increases the hydrophilicity as well as the size of the molecule, this latter approach also reduced renal filtration. Chen et al. (2004a, 2004c, 2004d, 2004e, 2004f) recently investigated this by comparing the imaging potential of PEGylated

Table 5
$\alpha_v\beta_3$ integrin-targeted radiotracer

Compound	Tumour model	Tumour uptake (%ID/g)	Tumour/muscle	Tumour/blood	Reference
[^{125}I]-Gluco-RGD	M21 melanoma[a]	1.81 ± 0.30, 120 min p.i.	6.2	7.9	Haubner et al. (2001a)
[^{18}F]-Galacto-RGD	M21 melanoma[a]	1.49 ± 0.10, 120 min p.i.	10.2	29.8	Haubner et al. (2001b)
[^{99m}Tc]-DKCK-RGD	M21 human melanoma[a]	1.08 ± 0.14, 120 min p.i.	8.1	9.7	Bock et al. (2000), Haubner et al. (2004)
[^{99m}Tc]-Hynic-E-c(RGDfK)$_2$	OVCAR-3 ovarian carcinoma[a]	5.6 ± 0.5[b], 120 min p.i.	4[b]	10[b]	Janssen et al. (2002a, 2002b)
[^{111}In]-DOTA-E-c(RGDfK)$_2$	OVCAR-3 ovarian carcinoma[a]	7.5 ± 1.5, 120 min p.i.	15[b]	35[b]	Janssen et al. (2002a, 2002b)
[^{18}F]-FB-c(RGDyK)	U87MG glioblastoma[a]	1.5 ± 0.3, 120 min p.i.[b]	21	8.2	Chen et al. (2004e)
[^{18}F]-FB-(c(RGDyK))$_2$	U87MG glioblastoma[a]				Chen et al. (2004g)
[^{18}F]-FB-PEG-c(RGDyK)	U87MG glioblastoma[a]	2.56 ± 0.12, 120 min p.i.	28.4	23.3	Chen et al. (2004c)
[^{64}Cu]-DOTA-c(RGDyK)	MDA-MB-435 mammary carcinoma[a]	1.36 ± 0.10, 120 min p.i.	6.2	7.2	Chen et al. (2004f)
[^{64}Cu]-DOTA-(c(RGDyK))$_2$	MDA-MB-435 mammary carcinoma[a]	3.1 ± 0.6, 120 min p.i.[b]	8[b]	24[b]	Chen et al. (2004b)
[^{64}Cu]-DOTA-PEG-c(RGDyK)	U87MG glioblastoma[a]	1.8 ± 0.2, 120 min p.i.[b]	10[b]	90[b]	Chen et al. (2004a)
[^{99m}Tc]-HPMA copolymer-RGD4C	DU145 prostate carcinoma	4.6 ± 1.8 24 h p.i.	164	18	Mitra et al. (2005)
[^{111}In]-TA138	c-neu oncomouse model[a]	3.34 ± 24 h p.i.	Muscle and blood level below detection limit		Onthank et al. (2004)

[a] $\alpha_v\beta_3$ integrin positive tumour cells.
[b] Estimated from graphical presentation.

and non-PEGylated c(RGDyK) tracers. In addition, tracers containing a dimeric RGD-peptide were evaluated (Chen et al., 2004b, 2004g). As expected, dimeric c(RGDyK) tracers exhibited higher tumour uptake and better tumour retention than single peptide ligands. Furthermore, the dimeric peptides were predominantly excreted via the kidney, in contrast to hepatic elimination for the monomers. The synergistic effect of multivalent binding and altered pharmacokinetics resulted in improved imaging characteristic of the dimeric peptides. The other strategy, attachment of a PEG-chain to the lysyl residue of the peptide, also resulted in improved pharmacokinetic properties of the tracer peptides. While tumour levels were comparable for PEGylated and non-PEGylated constructs, a more rapid renal elimination was observed for ^{64}Cu-DOTA-PEG-RGD, thus improving tumour/blood and tumour/tissue ratios. The lessons learned from RGD-mediated targeting of radiotracers have also been transferred to radiotherapy approaches. For this purpose, the dimeric peptide DOTA-E-c(RGDfK)$_2$ was chelated with ^{90}Y (Janssen et al., 2002b). When tested in ovarian carcinoma, the compound showed a significantly improved antitumour effect.

Several other recent papers focus on multivalent RGD-peptides to improve tumour accumulation and carrier pharmacokinetics for imaging (Liu et al., 2001a, 2001b; Mitra et al., 2005; Thumshirn et al., 2003). Approximately 15 RGD4C peptide groups were appended together with ^{99}Tc tracer groups to an HPMA copolymer thus combining receptor targeting with enhanced permeability retention in solid tumours (Mitra et al., 2005). This multivalent tracer showed a greatly increased circulation time compared to previous radiotracers, leading to highest tumour/organ ratios at 72 h post injection. Apart form the EPR effect which was also observed for the non-targeted polymer, the RGD4C-equipped tracer displayed a 3-fold improved accumulation in the tumour.

A rather novel development is the employment of RGD-peptidomimetics in angiogenesis imaging. The RGD-peptidomimetic TA138 was conjugated to DOTA for use as a radioactive tracer, either in combination with ^{111}In, ^{90}Y or even other nuclides (Harris et al., 2003; Liu et al., 2003). The radioactively labelled peptidomimetic showed specific binding to $\alpha_v\beta_3$ when tested in vitro, and displayed similar pharmacokinetic properties as hydrophilic RGD-peptide tracers, combining renal elimination with appropriate tumour accumulation (Onthank et al., 2004). It also showed promising antitumour efficacy when tested in the C-neu oncomouse model, which is $\alpha_v\beta_3$ positive, as well as in the $\alpha_v\beta_3$ negative HCT colon cancer model (Mousa et al., 2005). The TA138 RGD-mimetic was also conjugated to gadolinium containing paramagnetic nanoparticles for the purpose of non-radioactive magnetic resonance imaging (Winter et al., 2003). When these particles were evaluated in a C32 melanoma tumour model positive for $\alpha_v\beta_3$ on the tumour cells, RGD-targeted particles showed enhanced imaging versus non-targeted particles (Schmieder et al., 2005). In contrast, no differences were observed between the two types of tracers in the $\alpha_v\beta_3$ negative Vx-2 carcinoma model in

rabbits (Winter et al., 2003). Thus, similar to radioactive tracer approaches, the detection of angiogenic blood vessels per se via molecular MRI imaging of $\alpha_v\beta_3$ requires further optimization of the tracer technology.

4. Concluding remarks

Targeted delivery of therapeutics and diagnostics to tumour vasculature is recognised as a powerful approach for treatment of cancer, since angiogenesis is essential for tumour growth and endothelial cells are genetically more stable and therefore less prone to develop resistance. The direct contact with the blood stream makes them accessible for small and large targeting conjugates. $\alpha_v\beta_3$-integrin is expressed on angiogenic but not on resting endothelial cell. This restricted expression profile makes it in ideal target. To exploit this characteristic for targeted drug delivery and imaging, $\alpha_v\beta_3$-integrin specific RGD-peptides have been introduced into proteins, polymers, liposomes, viruses and other gene delivery vehicles. The fact that both chemical and recombinant conjugation approaches are feasible, has led to widespread application of RGD-mediated delivery in the field of drug targeting.

It is of importance to discriminate between two basically different targeting approaches, which are intracellular delivery and extracellular delivery. Intracellular delivery implies uptake of the delivered compound into the target cell and is compulsory for gene delivery and highly beneficial for delivery of small molecule drugs and apoptotic peptides. Uptake, however, has been shown to depend on the valency of the RGD-constructs with multivalent constructs showing superior performance. Thus, research has lately been focused on polymers, liposomes, protein carriers and viruses, which are all modified with a high number of RGD peptides. A single high affinity peptide coupled to a single drug will not be taken up as efficiently and will therefore be less potent. This group of multivalent carriers combines several other advantageous characteristics. The multivalent binding leads also to higher binding affinity and the high molecular weight results in a prolonged half-life and passive retention in tumours. Taken together, targeting of angiogenic blood vessels is best achieved using multivalent constructs, which will therefore likely receive more attention in the future.

Extracellular delivery is sufficient for imaging strategies and required for targeting of most therapeutic proteins like TNFα or tTF, which should not be internalised for activity. These furthermore should not be modified extensively to maintain the therapeutic activity of the proteins. Therefore, single modification with only one RGD peptide per protein subunit is a rational approach. The RGD peptide conformation has to be of the RGD4C, RGD10 type or a peptide with similar affinity in order to gain a targeted protein with proper targeting features. Such RGD-modified proteins have been shown to have an improved therapeutic index as compared to the parental protein.

With ^{18}F-Galacto-RGD the first RGD-based imaging agent was tested recently in humans, demonstrating highly desirable pharmacokinetics and good imaging of $\alpha_v\beta_3$-expression (Beer et al., 2005; Haubner et al., 2005). Despite the success with ^{18}F-Galacto-RGD, radiolabelled multivalent RGD-constructs also are emerging in this field and might eventually replace the current compounds (Thumshirn et al., 2003; Wu et al., 2005). Still, ^{18}F-Galacto-RGD offers the unique possibility to identify patients with high $\alpha_v\beta_3$-expression in tumour cells and endothelium that may benefit from RGD-based therapies. Combining this diagnostic tool for patient selection with a well designed RGD-targeted therapeutic can be a strong weapon against a variety of malignancies.

Currently, clinical phase I trials of the conditionally replicative Adenovirus Δ24.RGD are in preparation. These will focus on glioma and ovarian cancer both known for high expression of $\alpha_v\beta_3$-integrin. Despite promising results, other RGD-targeted drugs have not reached clinical trials yet. One reason for this may be the fact that synthesis reproducibility is not well addressed in the studies performed so far. The fact that $\alpha_v\beta_3$-integrin expression is not homogeneous throughout tumour endothelium might in general be a dilemma for RGD-based therapies in the future. Strategies that combine different target receptors might overcome this problem (Eichhorn et al., 2004; Bender et al., 2004) as well as combination with current standard therapies. Attacking the tumour at different cell types or at cells in different activation stages is likely to empower targeted strategies to successfully catch cancer off guard.

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