New ways in RGD-peptide mediated drug targeting to angiogenic endothelium

Temming, Kai

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2007

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 17-12-2018
Rational Design of RGD-albumin conjugates for targeted delivery of the VEGF-R kinase inhibitor PTK787 to angiogenic endothelium

Kai Temming$^{1,2}$, Marie Lacombe$^2$, Roel Q. J. Schaapveld$^2$, László Orfi$^4$, Gyorgy Kéri$^5$, Klaas Poelstra$^4$, Grietje Molema$^3$, Robbert J. Kok$^{1,6}$

$^1$Department of Pharmacokinetics & Drug Delivery, Groningen University Institute of Drug Exploration, The Netherlands
$^2$KREATECH Biotechnology B.V., Amsterdam, The Netherlands
$^3$Medical Biology Section, Department of Pathology and Laboratory Medicine, University Medical Center Groningen, University of Groningen, The Netherlands
$^4$Department of Pharmaceutical Chemistry, Semmelweis University, Budapest, Hungary
$^5$Department of Medical Chemistry, Peptide Biochemistry Research Group of the Hungarian Academy of Sciences, Semmelweis University, Budapest, Hungary.
$^6$Present affiliation: Department of Pharmaceutics, Utrecht University, The Netherlands

Chapter 4

Abstract

**Linking the Unlinkable:** Many new chemical entities lack reactive groups for use in formation of reversible bonds, e.g. to conjugate them for targeted drug delivery purposes. We succeeded with the noncovalent coupling of a potent signal transduction inhibitor to a protein backbone by applying the platinum based Universal Linkage System. The resulting drug targeting conjugates offer new potential for cancer treatment with highly limited side effects.
Introduction

Angiogenesis, the formation of new blood vessels out of pre-existing capillaries, is a prominent feature during the pathogenesis of cancer. Antiangiogenic therapies are therefore extensively investigated for combating this disease [1]. The newest treatments consist of antibodies or kinase inhibitors that inhibit the signaling by VEGF, one of the most prominent angiogenic modulators [2]. Here we propose specific targeting of the VEGF receptor (VEGF-R) kinase inhibitor PTK787 to angiogenic vasculature in tumor tissue. Our conjugates will combine the action of a kinase inhibitor with specificity for disease-controlling target cells, which can result in a far more restricted action and thus improved safety and efficacy of the drug. This may be relevant in view of side-effects associated with VEGF-R inhibition, e.g. vomiting, hypertension and embolism [3-5].

We have developed three new classes of drug carriers consisting of human serum albumin (HSA), cyclic RGD-peptides and polyethyleneglycol (PEG) (Scheme 1, upper panel). HSA served as a biocompatible and biodegradable carrier with a low polydispersity, thus allowing characterization of the final macromolecular conjugates by mass spectrometry. HSA was equipped with cyclic RGD-peptides as targeting ligands that bind with high affinity to the target receptor \( \alpha_v \beta_3 \)-integrin [6] that is overexpressed on angiogenic endothelium. This restricted expression profile and the good accessibility of endothelial cells make it an ideal target for drug delivery purposes [7, 8]. We applied either a short alkyl linker that enables introduction of a high number of RGD-peptides in the carrier (RGD-HSA), or an extended polyethylene glycol linker that presents the RGD-peptide at the distal end of the PEG chain (RGDPEG-HSA) but leads to lower RGD incorporation. The use of such a PEG linker will furthermore affect the distribution of the conjugates by the stealth effect of PEG, and will increase the solubility and decrease the immunogenicity of the products [9, 10]. A third carrier was designed by combination of the short alkyl linker for RGD incorporation together with separately attached monofunctional PEG groups (RGD-HSA-PEG).

Materials & Methods

**Synthesis of PTK787-ULS.** Cis-[Pt(ethylenediamine)nitrato-chloride] (referred to as ULS-NO\(_3\)) was synthesized by reacting cis-[Pt(ethylenediamine)chlorido] (referred to as ULS) in (10 mg/ml in \(N,N'\)-dimethylformamide (DMF)) with one molar equivalent of AgNO\(_3\) (59 mM or 1 mg in DMF). Precipitated silver chloride was removed by centrifugation. 282.9 \(\mu\)L of the resulting ULS-NO\(_3\) solution (20.5 \(\mu\)mol) then were added to 200 \(\mu\)L (5.77 \(\mu\)mol) of the solution of PTK787 (10 mg/ml in DMF). The resulting solution was heated at 37°C for 24 h during which consumption of the drug starting material was monitored by analytical HPLC on a reversed phase Luna2 C18 column that was maintained at 40 °C. The mobile phase consisted of a binary solvent system of triethylammonium acetate (100 mM pH 5.0):acetonitrile 90:10 (solvent A) and triethylammonium acetate (100 mM pH 5.0):acetonitrile 30:70 (solvent B). The column was eluted at a flow rate of 1.1 mL/min. Compounds were eluted at a stepwise gradient (0 % B from 0-4 min; 0-46 % B from 4-17 min; 46-100 % B from 17-19 min; 100 % B from 25-27 min; 0 % B from 27-34 min). PTK787 eluted at 17.2 mL and PTK787-ULS at 12.06 mL. The solvents were removed under reduced pressure and taken-up in 50:50 DMF:water. Mass spectrometry analysis confirmed the presence of the target 1:1 drug:ULS species and \(^{195}\)Pt NMR and \(^1\)H NMR studies.
indicated that binding of PTK787 to cis-[Pt(ethylenediamine)dichloride] takes place via co-ordination of the N- donor of the pyridine ring contained in the drug to the Pt(II) metal center.

NMR spectra were recorded on a 300 MHz Bruker DPX-300 spectrometer.

\[ ^{195} \text{Pt NMR of ULS-NO}_3: \ 2075 \text{ ppm.} \]

\[ ^{195} \text{Pt NMR of PTK787-ULS: -2493 ppm.} \]

\[ ^{1} \text{H NMR of PTK787 (CD}_3\text{OD):} \ \delta = 4.64 \text{ (s, 2H, CH}_2, 7.33 \text{ (m, 4H, N(CHCH}_3)_2 \text{ and (CHCC)}_2, 7.82 \text{ (d, J = 8.63 Hz, 2H, CIC(CHCH}_3)_2, 7.89 \text{ (m, 2H, CIC(CHCH}_3)_2, 8.04 \text{ (d, J = 7.80 Hz, 1H, CHCHCCNH), 8.40 \text{ (d, J = 6.06 Hz, 2H, N(CHCH}_3)_2, 8.44 \text{ (d, J = 7.74 Hz, 1H, CHCHCCCH}_2)_2 ppm.} \]

\[ ^{1} \text{H NMR of PTK787-ULS (CD}_3\text{OD):} \ \delta = 2.57 \text{ (m, 2H, CH}_2\text{NH}_2, 2.65 \text{ (m, 2H, CH}_2\text{NH}_2, 7.36 \text{ (d, J = 8.84 Hz, 2H, (CHCC)}_2, 7.48 \text{ (d, J = 6.51 Hz, 2H, N(CHCH}_3)_2, 7.82 \text{ (d, J = 8.48 Hz, 2H, CIC(CHCH}_3)_2, 7.95 \text{ (t, J = 8.61 Hz, 2H, CIC(CHCH}_3)_2, 8.10 \text{ (d, J = 7.25 Hz, 1H, CHCHCCNH), 8.48 \text{ (d, J = 7.58 Hz, 1H, CHCHCCCH}_2, 8.61 \text{ (d, J = 6.65 Hz, 2H, N(CHCH}_3)_2 ppm.} \]

Signals of the CH\text{2} protons in PTK-ULS were not discernable due to overlap with the solvent signal at 4.86 ppm.

Mass spectrometry of PTK787-ULS (ESI\+): Theoretical m/z: 637.45 [M]. Detected masses m/z: 658 [M+Na\+]-H\+, 637 [M]+, 601 [M-Cl-]-H\+, 637 [M]+. UV/Vis (in PBS): λ\text{max} 339 nm (ε = 11679 M\text{-1} cm\text{-1}).

Data were evaluated to be of a sufficient quality for conjugation to HSA with the crude product, and further purification of the species after conjugation to the macromolecular carrier.

**Synthesis of RGD-HSA.** HSA (30 mg, 444 nmol) dissolved in PBS was incubated with a 22-fold molar excess of iodoacetic acid N-hydroxysuccinimide ester (SIA-linker, SIGMA, MO, USA; 10mg/ml in DMF, 9.7 µmol). Meanwhile, the RGD-peptide c(RGDf(ε-S-acetylthioacetyl)K) (Ansynth Service, Roosendaal, The Netherlands) was dissolved at 10 mg/ml in a 1:4 acetonitrile/water mixture. The peptide (11.1 µmol) was added drop wise to the reaction mixture at a peptide to protein molar ratio of 25:1, after which hydroxylamine was added to a final concentration of 50 mM. Hydroxylamine will release the acetyl group of the RGD-peptide to obtain a free sulfhydryl group. The reaction was carried out over night at room temperature while protected from light after which the product was extensively dialyzed against PBS. The final product RGD-HSA was stored at -20°C. A control conjugate RAD-HSA was prepared according to the same protocol with the control peptide c(RADf(ε-S-acetylthioacetyl)K).

**Synthesis of RGD-HSA-PEG.** RGD-HSA (13 mg, 193 nmol) dissolved in PBS was incubated with a 20-fold molar excess of mPEG succinimidyl α-methylbutanoate (mPEG-SMB, Nektar Therapeutics, USA; 20 mg/ml, 3.85 µmol) and incubated for 3h at room temperature. The product was purified by size exclusion chromatography (SEC) using a Superdex200 HR 10/60 column on an Äkta System (GE Healthcare, Uppsala, Sweden). SEC was performed with 0.5 ml/min PBS and monitored at 214 nm, 280 nm and 339 nm. The final products RGD-HSA-PEG and RAD-HSA-PEG (prepared according to the same protocol) were stored at -20°C.

**Synthesis of RGDPEG-HSA.** HSA (10 mg, 148 nmol) was dissolved in PBS and incubated with a 50-fold molar excess of vinylsulfone-polyethylene glycol-N-hydroxysuccinimide ester (VNS-PEG-NHS; Nektar, Alabama, USA; 20 mg/ml in water, 7.4 µmol). The mixture was protected from light with aluminum foil and incubated for 1 h at room temperature while gently shaking on a spiramix roller bank. RGD-peptide (8.14 µmol) was added in a 55-fold molar excess over HSA followed by hydroxylamine addition as described above. Reaction was carried out over night at room temperature while protected from light. Remaining VNS groups were quenched by addition of cysteine (8.14 µmol; 55-fold molar excess over the amount of HSA), after which the
RGD-albumin conjugates for targeted delivery of PTK787

The final products RGDPEG-HSA and RADPEG-HSA were stored at -20°C.

**Scheme 1.** Schematic presentation of synthesis of drug targeting conjugates.

**Coupling of PTK787-ULS.** RGD-modified carriers dissolved in PBS were incubated with 15-fold molar excess of PTK787-ULS for 24 h at 37 °C, after which non-reacted PTK-ULS and
aggregated product was removed by SEC as described above. The products were sterilized by filtration via a 0.2 µm filter and stored at -20 °C.

Characterization of Synthesis Products. Protein content was evaluated using BCA protein kit (Pierce). Conjugation of PTK787 was quantified by UV-analysis at 339 nm, a specific wavelength of PTK787-ULS that is not found for HSA or free PTK787 (Figure S1). The molar absorption of PTK-ULS $\varepsilon_{339}$ (11679 M$^{-1}$cm$^{-1}$) was determined by spectrophotometric analysis of a known concentration of PTK787-ULS. Further spectra were taken at the beginning of the synthesis and after purification of drug targeting conjugates to determine the number of PTK787-ULS bound. Both the intermediate and the final products were subjected to analytical SEC to reveal increase in size, purity and grade of aggregation (data not shown). The number of RGD and PEG moieties coupled per HSA was determined by MALDI-TOF analysis using a Voyager-DE PRO workstation (Applied Biosystems). HSA, RGD-HSA, RGD-HSA-PEG and RGDPEG-HSA were dissolved at a concentration of 1 mg/ml in 50:50:0.1 methanol to water to acetic acid. A 1 µL aliquot was mixed with 1 µL of matrix (20 mg/ml sinapinic acid in 60:40:0.1 water to acetonitrile:trifluoroacetic acid), transferred onto a stainless steel sample holder and dried before being introduced into the mass spectrometer. Mass spectra were obtained by averaging the signals from 100 laser shots. Spectra were calibrated using bovine serum albumin (BSA) as a control. MALDI-TOF analysis of BSA provided among others signals of single charged monomeric BSA and of the single charged dimer of BSA. Our products displayed masses that were in-between these two calibration peaks. The number of RGDPEG groups bound per albumin was calculated from the averaged masses of the products.

Cells. HUVEC were obtained from the UMCG Endothelial Cell Facility. Primary isolates were cultured in 1 % gelatin-coated tissue culture flasks or culture wells (Corning, Costar, The Netherlands) at 37 °C under 5 % CO$_2$ / 95 % air. The culture medium, hereafter referred to as EC medium, consisted of RPMI 1640 (BioWittaker, Verviers, Belgium) supplemented with 20 % heat inactivated fetal calf serum (Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Invitrogen, Breda, The Netherlands), 5 U/ml heparin (Leo Pharmaceutical Products, Weesp, The Netherlands), 100 U/ml penicillin (Yamanouchi Pharma, Leiderdorp, The Netherlands), 100 µg/ml streptomycin (Radiumfarma-Fisiopharma, Italy), and 50 µg/ml endothelial cell growth factor supplement extracted from bovine brain. After attaining confluence, cells were detached from the surface by trypsin/EDTA (0.5/0.2 mg/ml in PBS) treatment and split in 1:3 ratio. Cells were used up to passage four.

Binding affinity of synthesis products. Binding affinity to $\alpha_v\beta_3$-integrin, expressed on the surface of HUVEC, was determined by competitive binding studies using $^{125}$I-labeled echistatin as radioligand for $\alpha_v\beta_3$-integrin. Echistatin was radiolabeled using the chloramine T method. On the day of the experiment, $^{125}$I-Echistatin was purified using a PD-10 column (GE Healthcare). Confluent monolayers of HUVEC in a 24well plate (Costar) were incubated with 100,000 cpm $^{125}$I-Echistatin in the presence of serial dilutions of drug targeting conjugates and control conjugates in binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM MnCl$_2$ and 1 % BSA) at 4 °C for 4 h. Subsequently, cells were washed three times with binding buffer and lysed with 1 M NaOH. Radioactivity in the lysate was counted in a Packard RIASTAR multiwell gamma counter (GMI, Minnesota, USA). Data were analyzed by non-linear regression using the GraphPad Prism program (GraphPad Software).
RGD-albumin conjugates for targeted delivery of PTK787

Table 1: Characteristics of RGD-peptide and PEG modified albumins as determined based on MALDI-TOF data (n.a.: not applicable)

<table>
<thead>
<tr>
<th>Product</th>
<th>RGD(PEG) : HSA</th>
<th>PEG : HSA</th>
<th>Molecular Size in kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGD-HSA</td>
<td>13 : 1</td>
<td>n.a.</td>
<td>75.9</td>
</tr>
<tr>
<td>RGD-HSA-PEG</td>
<td>13 : 1</td>
<td>1.6 : 1</td>
<td>85.1</td>
</tr>
<tr>
<td>RGDPEG-HSA</td>
<td>7 : 1</td>
<td>7 : 1</td>
<td>95.9</td>
</tr>
</tbody>
</table>

Figure 1. MALDI-TOF characterization of RGD-modified carriers. Peaks of single charged proteins are shown. Note the increase in size between starting material (A: HSA) and products (B: RGD-HSA; C: RGD-HSA-PEG; D: RGDPEG-HSA) that has been used to calculate the number of introduced groups.

**Effect of drug targeting conjugates on VEGF induced gene expression.** Effect of targeted PTK787 on VEGF induced gene expression was evaluated using quantitative real time PCR. In short, confluent monolayers of HUVEC in 12-well plates were incubated with drug targeting conjugate (concentration: 500 nM of the coupled PTK787), carrier alone (concentration: same as...
respective drug targeting conjugate) or non-conjugated drug (concentration: 100 nM) in EC medium containing 1.5 % FCS for 24 h followed by a challenge with 5 ng of VEGF for 50 min. Cells were washed twice with ice cold PBS and lysed. Total RNA was isolated with Absolutely RNA Microprep Kit (Stratagene, CA. USA) according to the manufacturer’s protocol. The amount of isolated RNA was quantified by NanoDrop. Equal amounts of RNA were transcribed to cDNA by SuperScript III First-Strand Synthesis System (Invitrogen). cDNA was diluted to 10 ng/μl concentration after RT-reaction. Exons overlapping primers and Minor Groove Binder (MGB) probes used for RT-PCR were purchased as Assay-on-Demand from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). Expression level of human NR4A1 was evaluated by RT-PCR on an ABI Prism 7900HT Sequence detection system (Applied Biosystems). Experiments were performed in triplicate from different HUVEC isolates.

**Statistical analysis.** Statistical analysis was performed using Student’s two-tailed t-test, assuming equal variances. Differences were considered to be significant when \( p < 0.05 \).

Figure 2. Typical UV-spectra of HSA, PTK-HSA, and RGDPEG-PTK-HSA demonstrating the specific absorbance of PTK787-ULS at 339 nm. This property was used for quantitation of drug bound to the carrier.

**Results and Discussion**

Previous studies have shown that it is of major importance to introduce multiple RGD-peptides in the conjugates to allow multivalent receptor interactions which facilitates binding and internalization by target cells \([11, 12]\). We therefore optimized the attachment of the targeting ligand, followed by subsequent conjugation of the drug. RGD coupling was achieved using a 22-fold molar excess of N-hydroxysuccinimide ester (SIA) or a 50-fold molar excess of vinylsulfone-polyethyleneglycol-N-hydroxysuccinimide ester (VS-PEG-NHS, 3.5 kDa) over HSA. The RGD-peptide c(RGD(ε-S-acetylthioacetyl)fK) was added in a slight excess over the added linker (25-fold respective 55-fold molar excess over HSA). After purification of the products RGD-HSA and RGDPEG-HSA, a fraction of RGD-HSA was further modified with monofunctional mPEG-SMB (5 kDa) in a ratio of 20:1 to obtain RGD-HSA-PEG. MALDI-TOF analysis elegantly demonstrated the incorporation of RGD and
RGD-albumin conjugates for targeted delivery of PTK787

PEG in the carriers (Figure 1, Table 1). Attachment of RGD to the compounds could be furthermore deduced from the binding studies with target cells (vide infra).

Table 2: Influence of PTK787 conjugation on solubility and recovery of drug targeting conjugates

<table>
<thead>
<tr>
<th>Product</th>
<th>PTK787 : HSA</th>
<th>Recovery in %&lt;sup&gt;[1]&lt;/sup&gt;</th>
<th>Monomeric fraction&lt;sup&gt;[2]&lt;/sup&gt; in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGD-PTK-HSA</td>
<td>6.3 : 1</td>
<td>43.5</td>
<td>48.5</td>
</tr>
<tr>
<td>RGD-PTK-HSA-PEG</td>
<td>7.6 : 1</td>
<td>66</td>
<td>83</td>
</tr>
<tr>
<td>RGDPEG-PTK-HSA</td>
<td>9.7 : 1</td>
<td>84.5</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>[1]</sup> in percent of starting amount of carrier.
<sup>[2]</sup> Monomeric fractions were determined before SEC purification. Final products did not contain aggregated materials.

The VEGF-R kinase inhibitor PTK787 contains none of the reactive groups that are commonly used for conjugation to a carrier. Employing a novel platinum(II)-based linker, to which PTK787 binds via a coordination linkage at one of the aromatic nitrogens, we nevertheless succeeded in the conjugation of this ‘unlinkable’ drug (Scheme 1, lower panel). PTK787 was appended to this so called Universal Linkage System (ULS™; cis-Pt(ethylenediamine)nitrato-chloride) by reacting at a 1:1 molar ratio. PTK787 was completely consumed during synthesis. Electrospray ionization mass spectrometry of the purified product confirmed the presence of 1:1 PTK787-ULS species, (MS (ESI) m/z: 658, 637 and 601. <sup>195</sup>Pt-NMR studies revealed a peak at -2493 ppm typical for N<sub>3</sub>Cl co-ordination involving two primary amines and one aromatic N-donor. Furthermore, shifts in the <sup>1</sup>H-NMR spectrum indicated that binding of ULS to PTK787 took place via coordination at the pyridyl ring. Protons in close proximity to the pyridyl nitrogen shifted from 7.33 to 7.48 ppm and from 8.4 to 8.61 ppm, while more distant protons where hardly affected. The drug-linker adduct demonstrated a specific UV-absorbance at 339 nm that was employed for the quantification of the drug carrier payload (Figure 2). To introduce an average of 5-10 drugs per HSA, PTK787-ULS was added in a 15-fold molar excess to the RGD-modified albumin carriers and the reaction was allowed to proceed for 24 h at 37 °C after which the macromolecular products were purified by size exclusion chromatography (SEC). During synthesis, clear differences were observed in the solubility of the products. Approximately 35 % of RGD-PTK-HSA precipitated and SEC revealed the presence of aggregates, most likely due to changes in charged groups and increased hydrophobicity at the carrier surface. In contrast, RGD-PTK-HSA-PEG that contains the same number of RGD per carrier did not precipitate nor did RGDPEG-PTK-HSA. This illustrates the rationale of incorporating PEG groups in RGD-HSA to improve solubility of the products. RGDPEG-PTK-HSA, containing seven PEG molecules acting as bifunctional linker, displayed highest drug:protein ratios as it neither precipitated nor aggregated. High drug/carrer payloads were achieved in all three conjugates since ULS does not aim for the same binding sites as the introduced alkyl linker or PEG linkers, which bound to primary amino groups. PTK787-ULS will form a coordination bond with sulfur containing residues, mostly methionines, but also with histidine residues as demonstrated earlier [13, 14]. Furthermore, we did
not observe steric hindrance of the bulky PEG moieties as similarly high drug/HSA ratios were found when PTK-ULS was reacted with native HSA (data not shown).

![Graph](image-url)

**Figure 3.** Binding to angiogenic endothelium. Binding affinity was detected by coincubation of \(^{125}\text{I}-\)echistatin, a known ligand for \(\alpha_\text{v}\beta_3\)-integrin, and increasing concentrations of drug targeting conjugates with HUVEC. A-C each display binding affinity of one drug targeting conjugate together with the respective RAD modified control conjugate and the RGD modified carrier without drug. In D all three drug targeting conjugates are compared.

Drug targeting conjugates and the respective control conjugates were analyzed for binding affinity to the \(\alpha_\text{v}\beta_3\)-integrin target receptor on human umbilical vein endothelial cells (HUVEC). Binding affinity was determined by competition studies with the well known \(\alpha_\text{v}\beta_3\)-integrin ligand \(^{125}\text{I}-\)echistatin and serial dilutions of conjugates. All RGD-equipped conjugates completely displaced \(^{125}\text{I}-\)echistatin, while RAD-conjugates were devoid of displacement capacity (Figure 3 A-C). Furthermore, conjugated PTK787 did not obstruct binding of the RGD-modified carriers to \(\alpha_\text{v}\beta_3\)-integrin (Figure 3 A-C). Although all conjugates displayed good binding characteristics to \(\alpha_\text{v}\beta_3\)-integrin, major differences could be observed among the different preparations (Figure 3 D). Highest binding affinity was determined for RGD-PTK-HSA (IC\(_{50}\): 4.4 nM; 0.3 g/ml) followed by RGD-PTK-HSA-PEG (IC\(_{50}\): 65 nM, 4.4 g/ml) and RGDPEG-PTK-HSA (IC\(_{50}\): 640 nM, 43 g/ml). This implies that a high number of RGD peptides inferred high avidity binding, but that additional incorporation of PEG partially obstructed binding. Nevertheless, RGD-PTK-HSA-PEG still bound with a 10-fold higher affinity than RGDPEG-PTK-HSA.
Figure 4. Effect of targeted delivery of PTK787 on VEGF induced gene expression. A confluent monolayer of HUVEC was incubated for 24h with drug targeting conjugates (conc.: 500 nM of PTK787), control conjugates (conc.: same as respective drug targeting conjugate) or drug (conc.: 100 nM) and thereafter challenged for 50 min with VEGF (5 ng/ml). Expression of hEGR3 (A), hNR4A3 (B) and hNR4A1 (C) were determined by quantitative RT-PCR. Differences between drug targeting conjugates and respective control conjugates were in all cases statistically significant (p < 0.05).

High binding affinity will positively influence the targeting ability of the conjugate, but the overall capability to reach target cells will also depend on other parameters like circulation time,
(non)specific binding and uptake by other organs. Since PEGylation will positively influence those parameters, it can not be predicted which of the products will display best delivery properties in vivo. The PEGylated drug carriers therefore remain highly interesting candidates in spite of lower binding avidity as compared to RGD-PTK-HSA.

Finally, drug targeting conjugates were tested for their ability to inhibit VEGF induced gene expression. EGR3, a zinc finger transcription factor, and the nuclear receptors NR4A1 and 3 were readily upregulated after addition of VEGF to endothelial cells. All three drug targeting conjugates were able to inhibit this upregulation significantly, while carrier without drug was devoid of activity (Figure 4). RGD-PTK-HSA was the most effective of the three compounds in spite of having the lowest drug to carrier ratio, corroborating the importance of high binding affinity for $\alpha_v\beta_3$-integrin targeted drugs as also reviewed recently [7, 15]. The drug targeting conjugates demonstrated less inhibition of gene expression compared to free PTK787, which can be explained by different mechanisms of uptake and the required processing of the conjugates by target cells. While the RGD-equipped macromolecular prodrugs require receptor mediated endocytosis and lysosomal processing for drug release [12, 16], free PTK787 can readily enter any cells by passive diffusion. This difference will favor the activity of the free drug in vitro, but at the same time will be a disadvantage in vivo compared to targeted drugs that comprise target cell specificity. We previously showed that the ULS-based coordination linkage is stable in serum, while drug is released upon competition with sulfur containing ligands like glutathione, of which high concentrations are found intracellularly [17, 18]. We expect a similar release mechanism for the PTK787-albumin conjugates. In future studies, we will investigate the targeting capability of these compounds and their tumor growth inhibitory properties.

The present series of RGD-equipped conjugates greatly expands the possibilities for specific intervention in dysregulated endothelium in cancer due to the rational combination of conventional linkers for RGD incorporation with the novel ULS linker for drug conjugation. Our drug-ULS linkage strategy is furthermore applicable to other drugs and carriers [17-19]. Optimization of the ligand targeted drug delivery conjugates by additional PEGylation not only improved pharmaceutical properties such as solubility and aggregation, but will also positively affect the in vivo behavior.

Acknowledgements

This work is supported by grants from Marie Curie (HPMI-CT-2002-00218), SenterNovem (TSGE1083) and Hungarian Scientific Research Fund OTKA (T-049478). We kindly acknowledge H. E. Moorlag from the endothelial cell facility UMCG for skillful isolation and culturing of cells and our colleagues at KREATECH for critical reviewing of the manuscript.

Reference List

RGD-albumin conjugates for targeted delivery of PTK787


