DOI: 10.1002/cmdc.200600201

Rational Design of RGD–Albumin Conjugates for Targeted Delivery of the VEGF-R Kinase Inhibitor PTK787 to Angiogenic Endothelium

Kai Temming,*[a, b] Marie Lacombe,[b] Roel Q. J. Schaapveld,[b] László Orfi,[e] Gyorgy Kéri,[f] Klaas Poelstra,[a] Grietje Molema,[d] and Robbert J. Kok[a, c]

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 block the signaling by VEGF, one of the most prominent angiogenic modulators.[2] Herein we propose specific targeting of the VEGF receptor (VEGF-R) kinase inhibitor PTK787 to angiogenic vasculature in tumor tissue. Our conjugates combine the action of a kinase inhibitor with specificity for disease-controlling target cells. This can result in a far greater restriction in drug action and thus an improvement in safety and efficacy. This may be relevant in view of side effects associated with VEGF-R inhibition, such as 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 polyethylene glycol (PEG) (Scheme 1 A). HSA served as a biocompatible and biodegradable carrier with 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] which is overexpressed on angiogenic endothelium. This restricted expression profile and the good accessibility of endothelial cells make them an ideal target for drug delivery.[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 which leads to lower RGD incorporation. The use of such a PEG linker furthermore affects the distribution of the conjugates by the stealth effect of PEG, and increases the solubility and decreases 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).

Previous studies have shown that it is very important to introduce multiple RGD peptides in the conjugates to allow multivalent receptor interactions to facilitate 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 carried out with a 22-fold molar excess of *N*-hydroxysuccinimide ester (SIA) or a 50-fold molar excess of vinylsulfone polyethylene glycol-*N*-hydroxysuccinimide ester (VS-PEG-NHS, 3.5 kDa) over HSA. The RGD peptide *c*(RGDf(ε-*S*-acetylthioacetyl)K) was added in 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 MS analysis elegantly demonstrated the incorporation of RGD and 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, as discussed below.

Table 1. Characteristics of RGD-peptide- and PEG-modified albumins determined by MALDI-TOF MS.

Product	RGD(PEG)/HSA	PEG/HSA	*M*, [kDa]
RGD-HSA	13:1	na[a]	75.9
RGD-HSA-PEG	13:1	1.6:1	85.1
RGDPEG-HSA	7:1	7:1	95.9

[a] Not applicable.

The VEGF-R kinase inhibitor PTK787 contains none of the reactive groups that are commonly used for conjugation to a carrier. By using a novel platinum(II)-based linker to which PTK787 binds through a coordination linkage at one of the aromatic nitrogen atoms, we nevertheless succeeded in the conjugation of this "unlinkable" drug (Scheme 1 B). PTK787 was appended to this so-called universal linkage system (ULS™, [Pt(*cis*-ethylenediamine)nitrate chloride]) by reaction of the two species at a 1:1 molar ratio. PTK787 was completely consumed during synthesis. Electrospray ionization mass spec-

[a] *K. Temming, Prof. Dr. K. Poelstra, Dr. R. J. Kok*
Department of Pharmacokinetics & Drug Delivery
Groningen University Institute of Drug Exploration
Antonius Deusinglaan 1, 9713 AV Groningen (The Netherlands)
Fax: (+31) 50-363-3247
E-mail: k.temming@rug.nl

[b] *K. Temming, Dr. M. Lacombe, Dr. R. Q. J. Schaapveld*
KREATECH Biotechnology B.V., Amsterdam (The Netherlands)

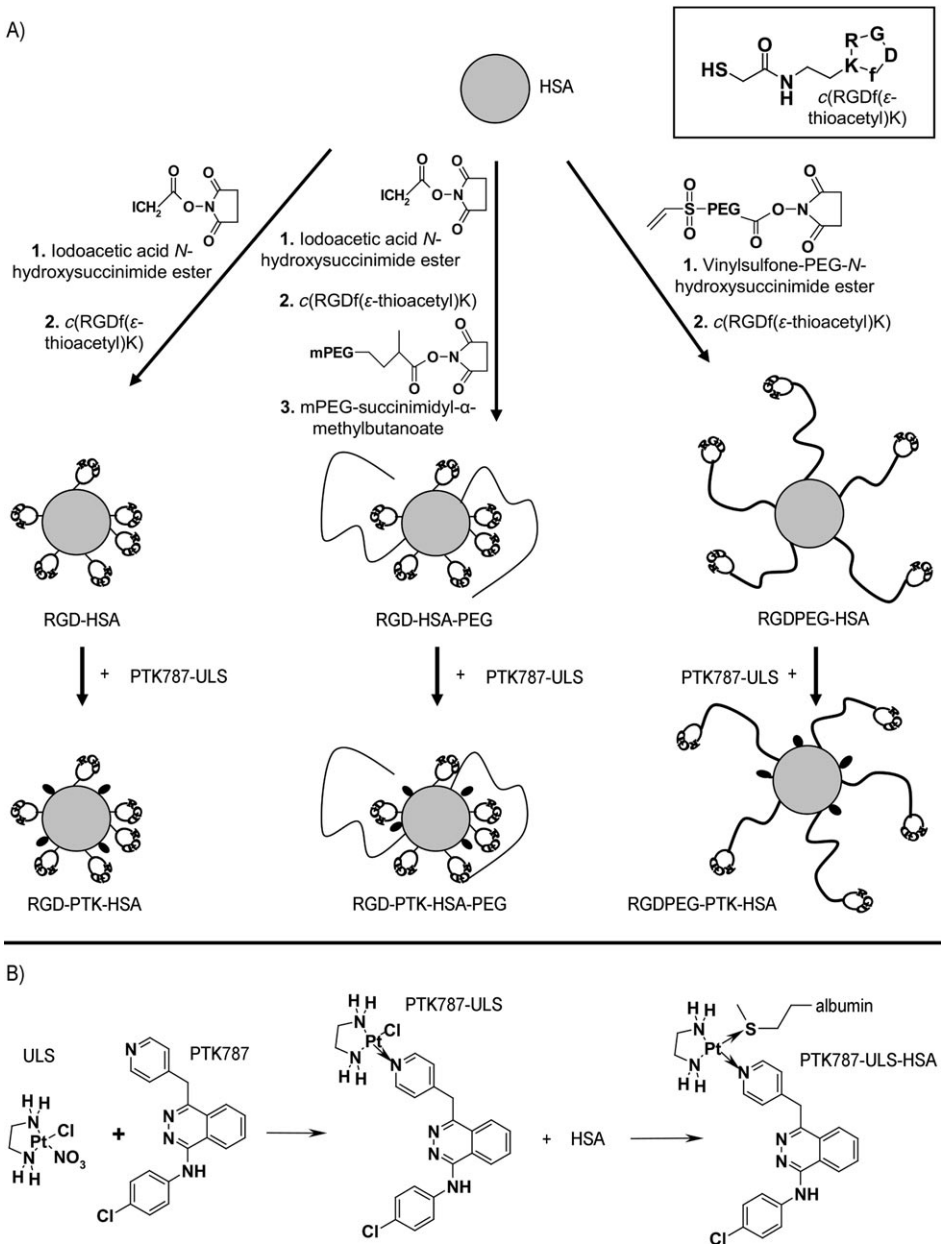
[c] *Dr. R. J. Kok*
present affiliation:
Department of Pharmaceutics, Utrecht University (The Netherlands)

[d] *Prof. Dr. G. Molema*
Medical Biology Section
Department of Pathology and Laboratory Medicine
University Medical Center Groningen
University of Groningen (The Netherlands)

[e] *Prof. Dr. L. Orfi*
Department of Pharmaceutical Chemistry
Semmelweis University, Budapest (Hungary)

[f] *Prof. Dr. G. Kéri*
Department of Medical Chemistry
Peptide Biochemistry Research Group of the Hungarian Academy of Sciences
Semmelweis University, Budapest (Hungary)

Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author.



Scheme 1. A) Synthesis of drug-targeting conjugates. B) Coupling of PTK787 to albumins.

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

Product	PTK787/HSA	Recovery [%][a]	Monomeric Fraction[b] [%][a]
RGD-PTK-HSA	6.3:1	43.5	48.5
RGD-PTK-HSA-PEG	7.6:1	66	83
RGDPEG-PTK-HSA	9.7:1	84.5	100

[a] Percentage of starting amount of carrier. [b] Monomeric fractions were determined before SEC purification; final products did not contain aggregated materials.

trometry of the purified product confirmed the presence of the 1:1 PTK787–ULS species, (MS (ESI^+) *m/z*: 658, 637, and 601). ^{195}Pt NMR studies revealed a peak at −2493 ppm, which is typical for a 3-N, 1-Cl coordination environment that involves two primary amines and one aromatic N donor. Furthermore, shifts in the ^1H NMR spectrum indicated that binding of ULS to PTK787 took place by coordination at the pyridyl ring. Protons in close proximity to the pyridyl nitrogen atom shifted from 7.33 to 7.48 ppm and from 8.40 to 8.61 ppm, whereas more distal protons where hardly affected. The drug–linker adduct demonstrated a specific UV/Vis absorbance at $\lambda = 339$ nm that was used for the quantitation of the drug/carrier payload (Supporting Information). To introduce an average of 5–10 drug molecules 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) (Table 2). 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, which contains the same number of RGD units per carrier did not precipitate, nor did RGDPEG-PTK-HSA. This illustrates the rationale of incorporating PEG groups in RGD-HSA to improve the solubility of the products. RGDPEG-PTK-HSA, which contains seven PEG molecules that act as bifunctional linker, displayed the highest drug/protein ratios, as it neither precipitated nor aggregated. High drug/carrier payloads were achieved in all three conjugates, as ULS does not target the same binding sites as the introduced alkyl or PEG linkers, which bind to primary amino groups. PTK787–ULS forms a coordinating bond with sulfur-containing residues, mostly methionines, but also with histidine residues, as has been demonstrated earlier.[13,14] Furthermore, we did not

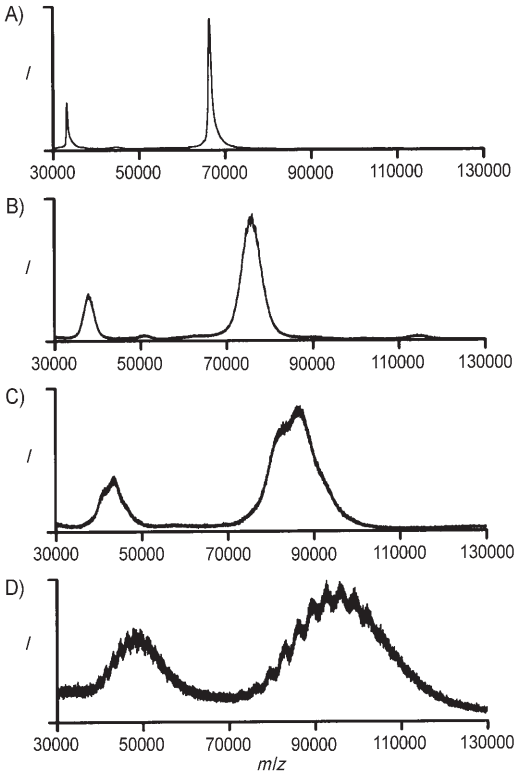


Figure 1. MALDI-TOF MS characterization of RGD-modified carriers: peaks of single charged proteins are shown. Note the increase in size between A) starting material (HSA) and products B) RGD-HSA, C) RGD-HSA-PEG, and D) RGDPEG-HSA, that was used to calculate the number of groups introduced.

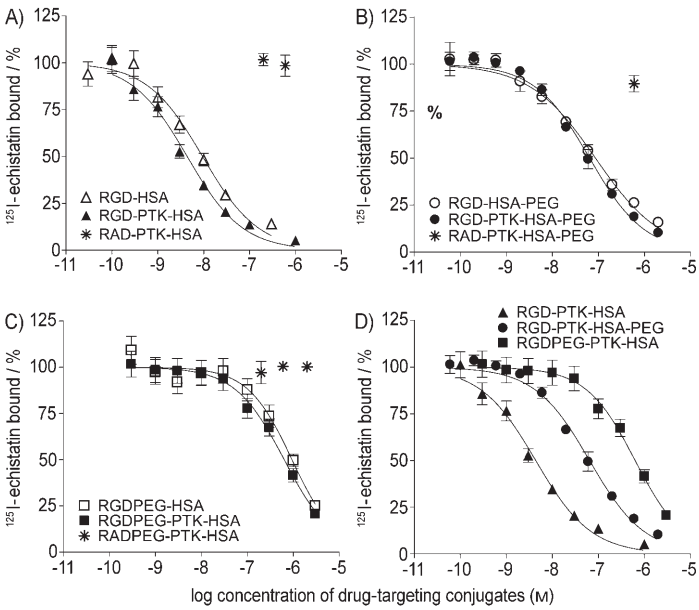


Figure 2. Binding to angiogenic endothelium: binding affinity was detected by co-incubation of ^{125}I-echistatin, a known ligand for $\alpha_v\beta_3$-integrin, and increasing concentrations of drug-targeting conjugates with HUVEC. A–C) Binding affinity of the indicated drug-targeting conjugate together with the respective RAD-modified control conjugate and the RGD-modified carrier without drug. D) Comparison of all three drug-targeting conjugates.

observe steric hindrance of the bulky PEG moieties, as similarly high drug/HSA ratios were found in reaction of PTK–ULS with native HSA (data not shown).

Drug-targeting conjugates and the respective control conjugates were analyzed for binding affinity to the $\alpha_v\beta_3$-integrin target receptor on human umbilical vein endothelial cells (HUVEC). Binding affinity was determined by competition studies with the well-known $\alpha_v\beta_3$-integrin ligand ^{125}I-echistatin and serial dilutions of conjugates. All RGD-equipped conjugates completely displaced ^{125}I-echistatin, whereas RAD conjugates were devoid of displacement capacity (Figure 2 A–C). Furthermore, conjugated PTK787 did not obstruct binding of the RGD-modified carriers to $\alpha_v\beta_3$-integrin (Figure 2 A–C). Although all conjugates displayed good binding characteristics to $\alpha_v\beta_3$-integrin, major differences were observed among the different preparations (Figure 2 D). The highest binding affinity was observed for RGD-PTK-HSA (IC_{50}: 4.4 nM, 0.3 $\mu g\,mL^{-1}$) followed by RGD-PTK-HSA-PEG (IC_{50}: 65 nM, 4.4 $\mu g\,mL^{-1}$) and RGDPEG-PTK-HSA (IC_{50}: 640 nM, 43 $\mu g\,mL^{-1}$). This implies that a high number of RGD peptides confers high-affinity binding, but that the additional incorporation of PEG partially obstructed binding. Nevertheless, RGD-PTK-HSA-PEG still bound with a 10-fold higher affinity than RGDPEG-PTK-HSA.

High binding affinity will positively influence the targeting ability of the conjugate, but the overall ability to reach target cells will also depend on other parameters such as circulation time, (non)specific binding, and uptake by other organs. As PEG modification will positively influence those parameters, it cannot be predicted which of the products will display the best delivery properties in vivo. The PEG-modified drug carriers therefore remain highly interesting candidates in spite of their lower binding avidity relative to that of 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 NR4A3 were readily upregulated after the addition of VEGF to endothelial cells. All three drug-targeting conjugates were able to inhibit this upregulation significantly, whereas carrier without drug was devoid of activity (Figure 3). RGD-PTK-HSA was the most effective of the three compounds, despite having the lowest drug/carrier ratio. This corroborates the importance of high binding affinity for $\alpha_v\beta_3$-integrin-targeted drugs, as has also been reviewed recently.[7,15] The drug-targeting conjugates demonstrated less inhibition of gene expression compared with 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 cell by passive diffusion. This difference favors the activity of the free drug in vitro, but at the same time is disadvantageous in vivo relative to targeted drugs that are target-cell specific. We previously showed that the ULS-based coordination linkage is stable in serum, while drug is released upon competition with sulfur-containing ligands such as 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

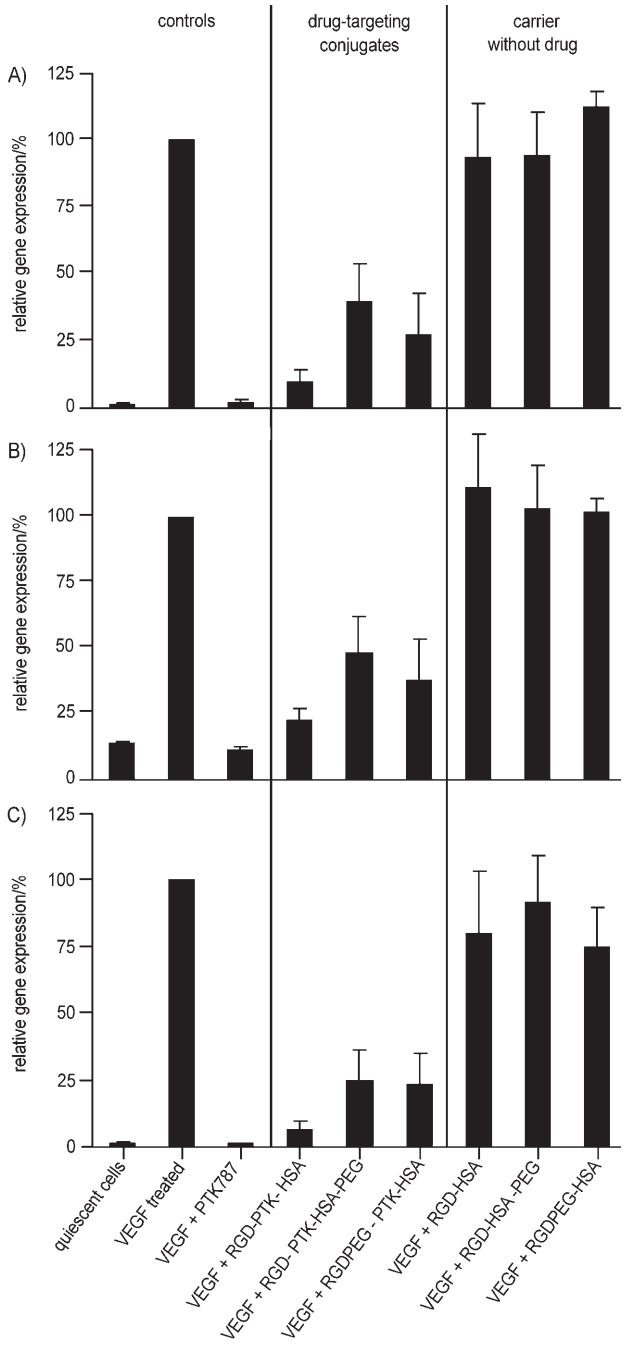


Figure 3. Effect of targeted delivery of PTK787 on VEGF-induced gene expression: a confluent monolayer of HUVEC was incubated for 24 h with drug-targeting conjugates (500 nM PTK787), control conjugates (concentration the same as respective drug-targeting conjugate), or drug (100 nM) and thereafter challenged for 50 min with VEGF (5 ng mL^{-1}). Expression of A) hEGR3, B) hNR4A3, and C) hNR4A1 were determined by quantitative RT-PCR. Differences between drug-targeting conjugates and respective control conjugates were statistically significant ($p < 0.05$) in all cases.

capacity 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 owing 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 PEG modification not only improved pharmaceutical properties such as solubility and aggregation, but will also positively affect the behavior in vivo.

Acknowledgements

This work is supported by grants from Marie Curie (HPMI-CT-2002-00218), SenterNovem (TSGE1083), and the 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.

Keywords: platinum · prodrugs · protein modifications · signal transduction · targeting

[1] N. Ferrara, R. S. Kerbel, *Nature* **2005**, *438*, 967 – 974.
[2] A. Zakarija, G. Soff, *Curr. Opin. Oncol.* **2005**, *17*, 578 – 583.
[3] A. L. Thomas, B. Morgan, M. A. Horsfield, A. Higginson, A. Kay, L. Lee, E. Masson, M. Puccio-Pick, D. Laurent, W. P. Steward, *J. Clin. Oncol.* **2005**, *23*, 4162 – 4171.
[4] B. C. Kuenen, M. Levi, J. C. Meijers, V. W. van Hinsbergh, V. J. Berkhof, A. K. Kakkar, K. Hoekman, H. M. Pinedo, *J. Clin. Oncol.* **2003**, *21*, 2192 – 2198.
[5] A. M. Levine, A. Tulpule, D. I. Quinn, G. Gorospe III, D. L. Smith, L. Hornor, W. D. Boswell, B. M. Espina, S. G. Groshen, R. Masood, P. S. Gill, *J. Clin. Oncol.* **2006**, *24*, 1712 – 1719.
[6] M. D. Pierschbacher, E. Ruoslahti, *Nature* **1984**, *309*, 30 – 33.
[7] K. Temming, R. M. Schiffelers, G. Molema, R. J. Kok, *Drug Resist. Updates* **2005**, *8*, 381 – 402.
[8] A. J. Schraa, M. Everts, R. J. Kok, S. A. Asgeirsdottir, D. K. Meijer, L. F. de Leij, G. Molema, *Biotechnol. Annu. Rev.* **2002**, *8*, 133 – 165.
[9] T. Tanaka, S. Shiramoto, M. Miyashita, Y. Fujishima, Y. Kaneo, *Int. J. Pharm.* **2004**, *277*, 39 – 61.
[10] P. Caliceti, F. M. Veronese, *Adv. Drug Delivery Rev.* **2003**, *55*, 1261 – 1277.
[11] R. J. Kok, A. J. Schraa, E. J. Bos, H. E. Moorlag, S. A. Asgeirsdottir, M. Everts, D. K. Meijer, G. Molema, *Bioconjugate Chem.* **2002**, *13*, 128 – 135.
[12] K. Temming, D. L. Meyer, R. F. Zabinski, E. C. F. Dijkers, K. Poelstra, G. Molema, R. J. Kok, *Bioconjugate Chem.* **2006**, submitted.
[13] M. C. Cox, K. J. Barnham, T. A. Frenkiel, J. D. Hoeschele, A. B. Mason, Q. Y. He, R. C. Woodworth, P. J. Sadler, *J. Biol. Inorg. Chem.* **1999**, *4*, 621 – 631.
[14] L. Trynda-Lemiesz, H. Kozlowski, B. K. Keppler, *J. Inorg. Biochem.* **1999**, *77*, 141 – 146.
[15] H. J. Wester, H. Kessler, *J. Nucl. Med.* **2005**, *46*, 1940 – 1945.
[16] A. J. Schraa, R. J. Kok, A. D. Berendsen, H. E. Moorlag, E. J. Bos, D. K. Meijer, L. F. de Leij, G. Molema, *J. Controlled Release* **2002**, *83*, 241 – 251.
[17] T. Gonzalo, E. G. Talman, A. van de Ven, K. Temming, R. Greupink, L. Beljaars, C. Reker-Smit, D. K. Meijer, G. Molema, K. Poelstra, R. J. Kok, *J. Controlled Release* **2006**, *111*, 193 – 203.
[18] K. Temming, M. Lacombe, P. van der Hoeven, J. Prakash, T. Gonzalo, E. C. F. Dijkers, L. Orfi, G. Keri, K. Poelstra, G. Molema, R. J. Kok, *Bioconjugate Chem.* **2006**, online publication ahead of print.
[19] J. Prakash, M. Sandovici, V. Saluja, M. Lacombe, R. Schaapveld, M. de Borst, H. van Goor, R. H. Henning, J. H. Proost, F. Moolenaar, G. Keri, D. K. F. Meijer, K. Poelstra, R. J. Kok, *J. Pharmacol. Exp. Ther.* **2006**, *319*, 1 – 12.

Received: August 14, 2006
Published online on September 22, 2006

