European Journal of Medicinal Chemistry 44 (2009) 3394–3401

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and biochemical evaluation of a cyclic RGD oxorhenium complex as new ligand of $\alpha_V\beta_3$ integrin

Marie Aufort, Marta Gonera, Nicolas Chaignon, Loïc Le Clainche, Christophe Dugave*

CEA/Saclay, iBiTec-S/SIMOPRO, Bâtiment 152, 91191 Gif-sur-Yvette, France

ARTICLE INFO

Article history:
Received 14 October 2008
Received in revised form
26 January 2009
Accepted 12 February 2009
Available online 27 February 2009

Keywords:
Integrin
RGD
Angiogenesis
Rhenium
Technetium
Cyclopeptide

ABSTRACT

We report the design of a new ligand of integrins that might be used for the molecular imaging of tumor neoangiogenesis. For this purpose, we designed a modified RGD tripeptide bearing a N-terminal *N*-bis(thioethyl)glycinate (NS_2) motif and a thioethyl moiety at the C-terminus. Simultaneous coordination of an oxorhenium core by the NS_2 and thioethyl moieties led to peptide cyclization and gave the corresponding monomers **13a** and **b** (major isomer) resulting from the *syn*/*anti*-isomerism, along with dimers' species **16a** and **b**. Cyclometallated peptide **13b** showed the most promising activity with an IC_{50} of 86 nM for integrin $\alpha_V\beta_3$ whereas it binds integrin $\alpha_{IIb}\beta_3$ with an affinity lower by an order of magnitude. Labeling with [^{99m}Tc]oxotechnetium gluconate led exclusively to complex **17**, the equivalent of compound **13b**, which displayed satisfactory stabilities in mice plasma and towards glutathione.

© 2009 Elsevier Masson SAS. All rights reserved.

1. Introduction

Integrins form a family of heterodimeric integral glycoproteins which play a central role in cell–cell adhesion and cell-matrix interactions [1,2]. In particular, integrin $\alpha_V\beta_3$ is implicated in tumor neoangiogenesis, cancer development and metastatic processes as well as acute renal failure [3–5]. The receptor recognizes a consensus RGD sequence of endogenous and exogenous proteins, such as the RGD-containing toxin echistatin [6], with a high selectivity which is essentially determined by the conformation of the RGD moiety [7].

Cyclopentapeptides such as cilengitide **1** [8–11], c(RGDfV) **2** and c(RGDyV) **3** [12], pyranne [13] and furane [14] derivatives (respectively **4** and **5**), cyclic amino acid derivatives **6–9** [15–18], cyclic disulfides [19] and thioether **10** [20,21] display high affinities for $\alpha_V\beta_3$ (Fig. 1). The cyclic molybdenum complex **11** was also proposed as an integrin antagonist [22]. Conversely, linear RGD sequences are weaker inhibitors [20,23,24]. Tracers derived from cyclopeptides **2** and **3** have been extensively used for the molecular imaging of integrin $\alpha_V\beta_3$ however, this strategy has reached some limitations in terms of structural and chemical diversities [25–42]. Therefore, we explored the potential of the metal-mediated peptide cyclization to build novel tracers with original structures.

Cyclization of peptides through metal coordination is an attractive strategy that confers interesting topologies and biochemical properties, as well as enhanced resistance to proteolysis [43–47]. Among metals used for this purpose, rhenium [47] and its artificial equivalent technetium [48,49] have been employed either to cross-bridge or to cyclize bioactive peptides. In particular, we recently showed that a proline-containing sequence cyclized through oxorhenium coordination binds to the human cyclophilin A (hCyp-18) with an affinity that is equivalent to that of linear substrates of hCyp-18. In this model, the oxorhenium core was coordinated by an NS_2/S complexation motif [50]. We applied this strategy to the design of an oxorhenium peptide conjugate, which was anticipated to bind integrin $\alpha_V\beta_3$, in order to evaluate the potential of this new class of integrin ligands. Moreover, synthesis of oxotechnetium equivalents of these complexes might lead to the development of new tracers usable for the molecular imaging of integrins.

The linear peptide **12** was designed from the canonic RGD sequence which was capped with a bis(thioethyl)glycinate (NS_2)

Abreviations: ClHOBT, 6-Chloro-1-hydroxy-1*H*-benzotriazole; DCC, Dicyclohexylcarbodiimide; DCM, Dichloromethane; DIPEA, Diisopropylamine; ES/MS, Electrospray mass spectrometry; GSH, Glutathione; HATU, 2-(7-Aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; LC–MS, Liquid chromatography coupled to a mass spectrometer; NMR, Nuclear magnetic resonance; PBS, Phosphate buffered saline; RGD, Arg-Gly-Asp; RP-HPLC, Reverse phase high performance liquid chromatography; SD, Standard deviation; SPPS, Solid phase peptide synthesis; TEA, Triethylamine; TFA, Trifluoroacetic acid; TIPS, Triisopropyl silane.

* Corresponding author. Tel.: +33 16908 5225; fax: +33 16908 9071.
E-mail address: christophe.dugave@cea.fr (C. Dugave).

0223-5234/$ – see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2009.02.022

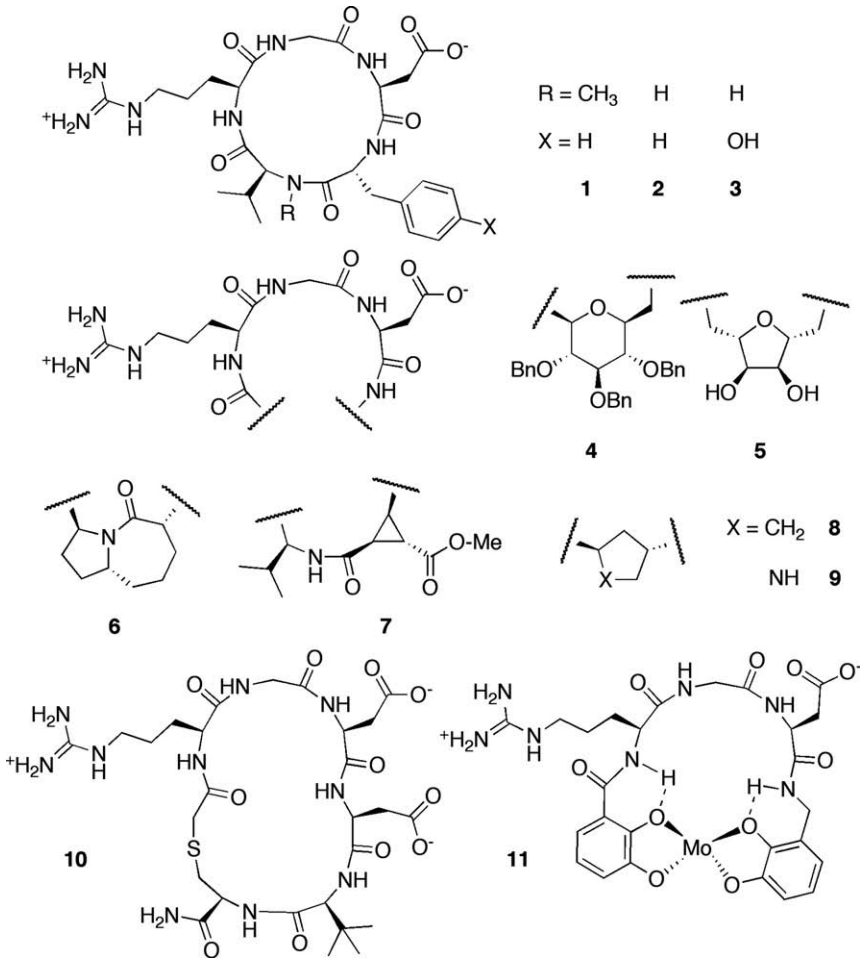


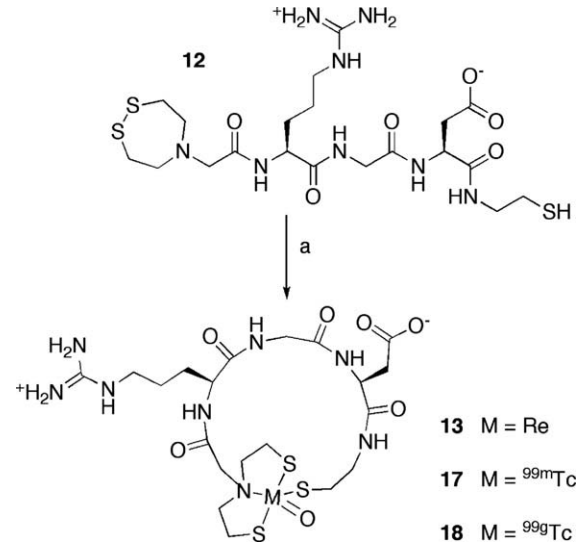
Fig. 1. Chemical structures of cyclic RGD analogs **1**–**10** and molybdenum-peptide conjugate **11**.

motif, while the C-terminus was grafted with a 2-aminoethanethiol motif (S moiety). Cyclization through the formation of $NS_2 \cdot MO \cdot S$ complexes **13** (M = ReO), **17** (M = ^{99m}TcO) or **18** (M = ^{99g}TcO) was assumed to restraint peptide flexibility (Scheme 1). We expected to obtain several isomeric complexes from a single peptide due to multiple *syn*/*anti*-isomerisms (tetrahedral nitrogen, position of the M=O motif relative to the peptide, see Scheme 2) [51,52] that might be separated and evaluated as ligands of integrins.

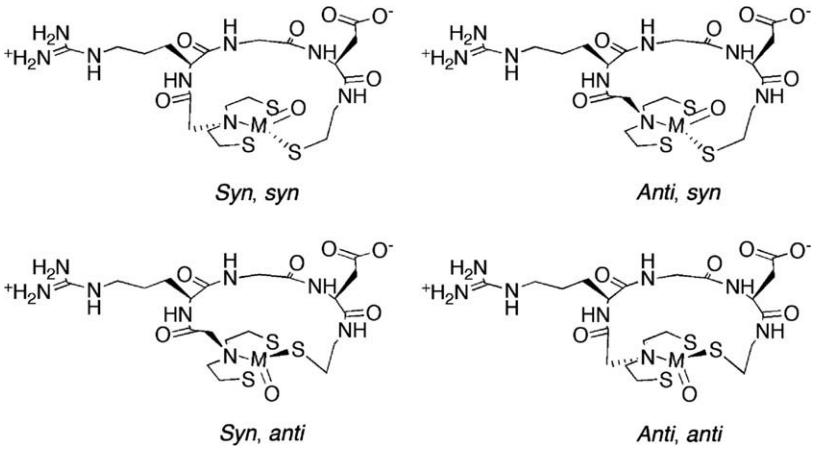
In this paper, we describe the synthesis and biochemical evaluation of cyclic metalloconstructs that result from metallation of peptide **12** with oxorhenium and oxotechnetium.

2. Material and methods

Chemical reagents and solvents were purchased from Sigma-Aldrich, VWR, Fluka or SDS and were of the highest purity available. Amino acids and resins were from Novabiochem. The preloaded Fmoc–Asp(PEG–PS)–allyl resin was purchased from Perseptive Biosystems Gmbh. HATU was purchased from Molekula (UK). [$^{99g}TcO_4$][NH_4] was obtained from the Oak Ridge National Laboratory and was purified before use. [$^{99m}TcO_4$]$^-$ was eluted as a physiological saline solution from commercially available ^{99}Mo/^{99m}Tc generator system (ELUMATIC III, CIS bio international). RP-HPLC purifications were achieved on a Prostar Varian chromatography system coupled to a Varian 335 diode array detector. RP-HPLC analysis was carried out on a Varian Pursuit C18 column (analytical column 250 × 4.6 mm, 5 μm) protected by an analytical Security Guard (Phenomenex). RP-HPLC purifications were performed either on a Varian Pursuit C18 preparative column (250 × 21.2 mm, 5 μm) or on a Varian Pursuit C18 semipreparative column (250 × 10.0 mm, 5 μm), eluted with various proportions of A and B



Scheme 1. Reagents and conditions: (a) 10% PBu_3/MeOH (1.5 equiv.) for 1 h then [$Bu_4NReOCl_4$] (1 equiv.) and TEA (4 equiv.) in MeOH for 2 h (**13**), [^{99m}TcO.gluconate$_2$] (depletion) in 35 mM HEPES buffer pH 7.8 (**17**) or [$Bu_4N^{99g}TcOCl_4$] (1 equiv.) (**18**).



Scheme 2. Schematic representation of 4 possible diastereomers formed through oxorhenium/oxotechnetium coordination by peptide **12** (M = Re/Tc). Influence of the $NS_2 \cdot ReO \cdot S$ configuration on the peptide conformation was not simulated.

(A, 0.1% aqueous solution of formic acid; B, 0.1% solution of formic acid in acetonitrile). The chromatography system was coupled to a gamma detector (radioflow monitor) HERM LB500 (Berthold). LC–MS analysis were carried out on an Agilent 1100 series chromatography system, with a Varian Pursuit C18 analytical column, with a linear gradient system (0–15% B) from 0 to 5 min, a linear gradient system (15–30% B) from 5 to 35 min and then an isocratic system (100% B). The chromatography system was coupled to a Bruker Esquire HCT ES/MS mass spectrometer. ^1H and ^{13}C NMR spectra were recorded on Bruker AVANCE 250 NMR spectrometer; δ and J are reported in ppm relative to TMS and Hz, respectively. High resolution mass spectrometry (HRMS) was performed on a Q-tof (Micromass). Microplates were counted using a Perkin–Elemer microplate scintillation and luminescence counter Topcount NXT.

2.1. S-triphenylmethyl-1-aminoethanethiol (trifluoroacetate salt) **14**

Triphenylmethanol (7.81 g, 30 mmol) and 2-aminoethanethiol hydrochloride (3.83 g, 1.1 equiv.) were stirred in pure trifluoroacetic acid (100 mL) for 2 h at room temperature. After evaporation of TFA, the residual oil was dissolved in DCM and the pH was raised to 9 by dropwise addition of 1 M sodium bicarbonate. Elimination of DCM under reduced pressure led to the formation of a white precipitate which was isolated by filtration to give **14** (12.85 g, 98,5%); ^1H NMR ($CDCl_3$, 250 MHz): δ 7.42–7.22 (m, 15H, CH Tr), 2.58 (t, 2H, $J = 6.5$, CH_2N), 2.26 (t, 2H, $J = 6.0$, CH_2S); ^{13}C NMR (D_2O, 62.5 MHz): δ 144.4 + 129.8 + 128.7 + 127.6 (Ar C), 67.9 (C Tr), 38.9 (CH_2N), 19.5 (CH_2S).

2.1.1. Compound **12** (as a monomer)

The preloaded Fmoc–Asp(PEG–PS)–allyl resin (loading 0.16 mmol g^{-1}, 1.25 g, 0.2 mmol) swelled in chloroform was treated with tetrakis(triphenylphosphine)palladium(0) (693 mg, 3.0 equiv.) in a mixture of chloroform/acetic acid/morpholine 37/2/1 (40 mL) for 2 h at room temperature. After elimination of the reagents, the resin was washed successively with 0.5% DIPEA in DMF, 0.5% sodium diethyldithiocarbamate in DMF, DMF, 8% DIPEA in DMF, DMF and DCM. Coupling with compound **14** (347 mg, 4 equiv.) was carried out in DMF with HATU (152 mg, 2 equiv.) and DIPEA (280 μL, 8 equiv.) overnight at room temperature. Elongation of the peptide was performed using a standard Fmoc peptide strategy with DCC and ClHOBT as coupling reagents in the presence of DIPEA in NMP. After removal of the N-terminal Fmoc group with 20% piperidine in NMP, the peptide was capped with compound **15** (5 equiv.) using DCC (5 equiv.), ClHOBT (5.5 equiv.) and DIPEA (5 equiv.) in NMP overnight at room temperature. After elimination of the reagents with NMP (×3), DCM (×3) and methanol (×3), the resin was dried and treated with a mixture of TFA/TIPS/water 95/2.5/2.5 for 3 h at room temperature. Filtration and precipitation of the filtrate in *tert*-butylmethyl ether and purification of the precipitate on a Varian Pursuit preparative column (20 mL mn^{-1}, isocratic (A/B 100/0) from 0 to 5 min, linear gradient (A/B 100/0 to 0/100) from 5 to 35 min and isocratic (A/B 0/100) for 5 min) gave **12**: HPLC $t_R = 12.9$ min. ^1H NMR (D_2O, 250 MHz): δ 8.63 (t, $J = 5.6$ Hz, 1H, NH), 8.37 (d, $J = 7.5$ Hz, 1H, NH), 8.21 (t, $J = 5.9$ Hz, 1H, NH), 4.66 (t, $J = 6.4$ Hz, 1H, CHα Asp), 4.38–4.33 (m, 1H, CHα Arg), 4.30 (s, 2H, CH_2 NS_2), 3.94 (s, 2H, CH_2 Gly), 3.88 (t, $J = 5.5$ Hz, 4H, 2 × CH_2–N NS_2), 3.37 (t, $J = 6.4$ Hz, 2H, N–CH_2–C–S), 3.25–3.16 (m, 6H, 2 × CH_2–S NS_2 and CH_2–N Arg), 2.88–2.84 (m, 2H, CH_2 Asp), 2.61 (t, $J = 6.4$ Hz, 2H, N–C–CH_2–S), 1.90–1.60 (m, 4H, $CH_2\beta$ and $CH_2\gamma$ Arg); ^{13}C NMR (D_2O, 62.5 MHz): δ 174.8 (COOH Asp), 174.4 (C═O Arg), 172.9 (C═O Asp), 171.6 (C═O Gly), 165.9 (C═O NS_2), 157.4 (C═N Arg), 58.9 (2 × CH_2–N NS_2), 58.9 (CH_2 NS_2), 54.4 (Cα Arg), 50.9 (Cα Asp), 43.0 + 42.9 (CH_2 Gly and N–C–C–S), 41.1 (CH_2–N Arg), 36.3 (CH_2 Asp), 34.5 (2 × CH_2–S NS_2), 28.8 (Cβ Arg), 24.9 (Cγ Arg), 23.8 (N–C–C–S). ES/MS (positive ionization): m/z 581.16 (MH^+, 22%), 291.25 ($\overline{M}2H^+$, 100%). HRMS: m/z calcd for $C_{20}H_{37}N_8O_6S_3$ 581.1998; found 581.2028.

2.2. Reduction of peptide **12**

Peptide **12** (100 μmol, 1 equiv.) was dissolved in degased methanol (1.5 mL). A solution of tributylphosphine 10% in methanol (150 μmol, 1.5 equiv.) was added and the mixture was stirred for 1 h at room temperature under argon. The solution was used immediately.

2.3. Rhenium complexes **13a**, **b**, **16a** and **b**

Tetrabutylammonium tetrachlorooxorhenate (100 μmol, 1 equiv.) in degased methanol (1.5 mL) followed by TEA (400 μmol, 4 equiv.) was added to the freshly prepared solution of reduced peptide **12**. The pH was adjusted to pH 10 with TEA. The mixture was stirred for 2 h at room temperature. The precipitate was isolated by centrifugation and purified by semipreparative RP-HPLC (5 mL mn^{-1}) with a linear gradient system (0–15% B) from 0 to 5 min, a linear gradient system (15–30% B) from 5 to 35 min and then an isocratic system (100% B). Purity of the compounds was checked by analytical RP-HPLC at 1 mL mn^{-1} (same gradient): $t_R = 11.7$ min (**13a**), 15.2 min (**13b**), 17.3 (**16a**) and 21.6 (**16b**).

LC–MS, compounds (t_R, ES/MS *m/z* main peaks): **13a** (10.0, 783.1 + 781.1 (MH^+)); **13b** (13.5, 783.1 + 781.1 (MH^+)); **16a** (15.9, traces, 387.2 ($M3H^+$) + 783.1 ($M2H^+$) + 1159.4 (MH^+)); **16b** (20.3, 387.2 ($M3H^+$) + 783.1 ($M2H^+$) + 1159.4 (MH^+)).

2.3.1. [^{99m}Tc]Technetium complex **17**

After reduction of peptide **12** (2 μmol) as described above, the solvent was removed under an argon flux and the product was dissolved in a 35 mM HEPES buffer pH 7.8 to give a 500 μM solution. [^{99m}Tc]oxotechnetium gluconate was produced by mixing solutions of 2 mg mL^{-1} stannous chloride (2 μL) in HCl 0.1 N, 50 mM gluconate (10 μL), and a [^{99m}Tc]pertechnetate solution (90 μL, about 130 MBq) eluted from a ^{99}Mo/^{99m}Tc generator. The resulting [^{99m}Tc]oxotechnetium gluconate solution was treated 15 min at 90 °C with the reduced peptide (100 μL).

Complex **17**: analytical RP-HPLC with a linear gradient system (0–15% B) from 0 to 5 min, a linear gradient system (15–30% B) from 5 to 35 min and then an isocratic system (100% B): $t_R = 14.5$ min.

2.3.2. [^{99g}Tc]Technetium complexes **18** and **19**

A methanolic solution (10 μL/μmol) of tetrabutylammonium tetrachlorooxo [^{99g}Tc]technetate (1 equiv.), prepared from [$^{99g}TcO_4$][NH_4] as previously described [53,54], was added to the solution of reduced peptide **12**. The mixture was vortexed to give a brown-red precipitate.

LC–MS analysis: compound **18**, $t_R = 14.3$ min (*m/z* 695.1, M + H^+); **19**, $t_R = 20.3$ min (*m/z* 695.1, M + H^+).

Analytical HPLC (same conditions as for **17**): $t_R = 14.2$ min (**18**) and 20.1 min (**19**).

2.4. Resistance of complex **17** to GSH

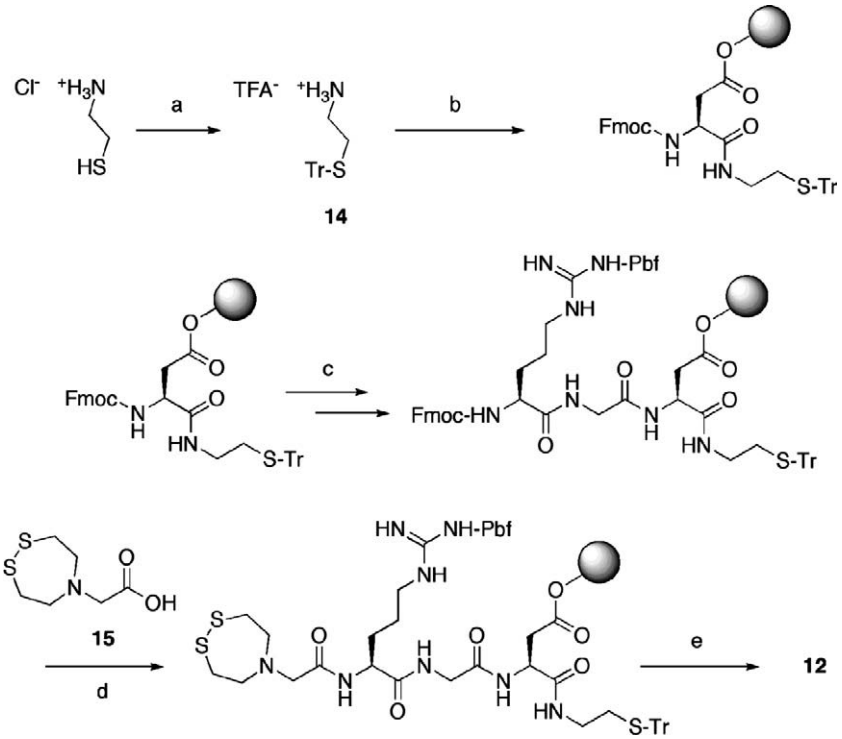
A solution of complex **17** (200 μL, 290 MBq) was added to a degased HEPES buffer (25 μL, 35 mM, pH 7.8) followed by a 10 mM solution of GSH in HEPES buffer (25 μL). The mixture was incubated at room temperature. Aliquots (10 μL) were diluted with water (990 μL) after 0, 0.5, 1, 2, 4 and 6 h and were analyzed by analytical RP-HPLC with an analytical column with an isocratic system (100% A) from 0 to 5 min, a linear gradient system (from 100% A to 100% B) from 5 to 35 min and then an isocratic system (100% B).

2.4.1. Stability of complex **17** in murine plasma

A solution of complex **17** (25 μL, 36 MBq) was added to freshly prepared murine plasma (125 μL) and the mixture was incubated at 37 °C. Aliquots (10 μL) were isolated after 0, 0.5, 1, 2, 4 and 6 h. Proteins were precipitated with methanol (90 μL) and separated by centrifugation. The supernatant was analyzed by analytical RP-HPLC (same gradient as above).

2.5. Integrin binding assays

$\alpha_V\beta_3$ integrin receptors (Chemicon, Temecula, CA) and $\alpha_{IIb}\beta_3$ integrin receptors (Enzyme Research, Swansea, UK) were diluted at 1000 ng/mL in coating buffer (20 mM Tris HCl pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$) and an aliquot of 100 μL/well was added to a 96-well plates (Millipore, Multiscreen-IP HTS) and incubated overnight at 4 °C. The plate was washed with the blocking/binding buffer (50 mM Tris HCl pH 7.4, 100 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and respectively 1 and 5% Bovine serum albumin for $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$) and incubated an additional 2 h at room temperature. Then the plate was incubated in the presence of varying amounts of competing ligand (0.1 nM to 10 μM RGD peptide) for 3 h with (0.06 nM) [^{125}I] I-echistatin. After incubation wells were washed two times with the blocking/binding buffer and counted by liquid scintillation method. Each data point is the result of the average of triplicate wells.



Scheme 3. Reagents and conditions: (a) TFA, TrOH (1 equiv.), 2 h; (b) Fmoc–Asp(PEG–PS)–OH resin, **14** (4 equiv.), HATU (2 equiv.), DIPEA (8 equiv.) in NMP, overnight; (c) SPPS; (d) 20% piperidine/NMP (X3) then **15** (5 equiv.), DCC (5 equiv.), ClHOBT (5.5 equiv.), DIPEA (5 equiv.) in NMP, overnight; (e) TFA, TIPS, H_2O 95/2.5/2.5.

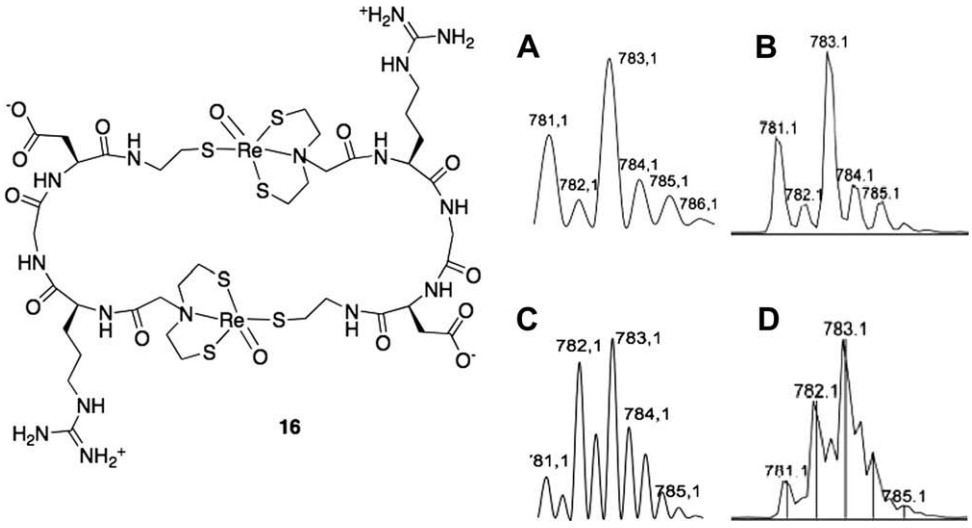


Fig. 2. Chemical structure of the homodimer complex **16** and comparison of ES/MS isotopic motifs (positive mode) corresponding respectively to monomer **13** (A, B) and homodimer **16** (C, D): theoretical (A, C) and experimental (B, D) isotopic profiles. Similar patterns B and D were observed for the two isomers of **13** and **16** respectively.

3. Results and discussion

The linear RGD peptide **12** was synthesized starting from a commercial PEG–PS resin grafted with Fmoc–Asp–OAllyl (Fmoc–Asp(PEG–PS)–OAllyl) as depicted in Scheme 3. Removal of the allyl group with tetrakistriphenylphosphine palladium(0) [55] and coupling with *S*-trityl-2-aminoethanethiol **14** with HATU afforded the corresponding Fmoc–Asp(PEG–PS)–NH–CH_2–CH_2–STr derivative. Compound **14** was obtained in almost quantitative yield as a trifluoroacetate (TFA) salt by simply stirring a mixture of triphenylmethanol and 2-aminoethanethiol hydrochloride in pure TFA for 2 h (Scheme 3). Classical peptide elongation by stepwise solid-phase peptide synthesis using dicyclohexyl-carbodiimide and 6-chloro-1-hydroxy-1*H*-benzotriazole as coupling reagents gave the polymer-supported peptide Fmoc–Arg(Pbf)–Gly–Asp(PEG–PS)–NH–$(CH_2)_2$–STr. After removal of the Fmoc protecting group, the peptide was capped with bis(thioethyl)glycinate **15**, prepared as previously reported [50]. Simultaneous resin cleavage and peptide deprotection in TFA (95%) with water (2.5%) and triisopropyl silane (2.5%) gave the free peptide **12** (Scheme 3) which was purified by RP-HPLC and identified by ES/MS and NMR spectroscopy.

Reduction of peptide **12** with tributylphosphine in methanol followed by reaction with tetrabutylammonium tetrachlorooxorhenate gave the corresponding oxorhenium complex (Scheme 1) which precipitated. The LC–MS analysis confirmed the presence of the rhenium complex as a mixture of *syn*/*anti*-diastereomers **13a** and **b** ($t_R = 10.0$ and 13.5 min respectively) along with the corresponding dimers **16a** and **b** ($t_R = 15.9$ and 20.3 min respectively, see Fig. 2). Complexes **13a**, **b** and **16b** were separated and collected by RP-HPLC at the milligram scale, and were evaluated as ligands of integrin $\alpha_V\beta_3$ using the standard competition assay with [^{125}I]echistatin [56]. Dimer **16a** (traces) could not be isolated in sufficient quantity and therefore, was not tested.

The standard competition assay showed that rhenium complex **13b** inhibited the binding of [^{125}I]echistatin to integrin $\alpha_V\beta_3$ with an IC_{50} of 86 nM which is equivalent to that of cyclopentapeptide antagonists such as compound **3** (Table 1) [12]. Conversely, diastereomer **13a** displayed a weaker affinity ($IC_{50} = 930$ nM) for the protein. The homodimer **16b** also displayed an interesting IC_{50}, though dimerization tends apparently to decrease the affinity for integrin $\alpha_V\beta_3$ relative to the monomer **13b**. This result might suggest that relaxation of constraints inside the dimer complex is deleterious for the biochemical activity. This hypothesis seems to be confirmed with the acyclic peptide **12** which displayed an IC_{50} of 360 nM for integrin $\alpha_V\beta_3$.

The compounds were also tested as ligands of $\alpha_{IIb}\beta_3$, another important integrin [1,2], and displayed lower affinities. In particular, IC_{50} of complexes **13b** and **16b** (respectively 1.1 μM and 4.5 μM) indicates that these compounds exhibit a preference for $\alpha_V\beta_3$ relative to $\alpha_{IIb}\beta_3$.

In order to check that the complexes are stable in the assay conditions, the solutions of complexes used for IC_{50} determinations were analyzed by LC–MS. Chromatograms indicated that complex **13b** is reasonably stable since no other rhenium complex and side-product could be detected except some traces of isomer **13a** (Fig. 3B). In contrast, isomer **13a** tends to isomerize slowly and gives isomer **13b** (5%) (Fig. 3A). Not surprisingly, dimer **16b** revealed to be more instable since it dissociated to give a mixture of **13a** (traces), **13b** (25%) and **16a** (20%) (Fig. 3C). These results must be considered to reevaluate the significance of IC_{50} calculated from the integrin binding assays since the apparent affinity of complexes **13a** and **16b** might be directly related to the presence of **13b**. In these conditions, it is difficult to conclude on the real biochemical activity of monomer **13a** and dimer **16b**, whereas isomer **13b** has an interesting affinity for $\alpha_V\beta_3$.

We investigated the evolution of the mixture of monomers **13a** and **b** and dimers **16a** and **b** after varying periods of time. For this purpose, the product of oxorhenium coordination by peptide **12** (mixture of **13** and **16**) was incubated in a 1X PBS buffer pH 7.4 at

Table 1
IC_{50} (nM ± SD) for the competition experiments of compounds **3**, **12**, **13a**, **b** and **16b** with [^{125}I]echistatin in the $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ binding assays.

Compounds	Products of isomerization and dimerization[a]	$\alpha_V\beta_3$[b]	$\alpha_{IIb}\beta_3$[b]
3	–	67 ± 35	675 ± 60
12	**12** homodimer (100%)[c]	360 ± 220	930 ± 85
13a	**13b** (5%)	930 ± 65	3270 ± 440
13b	Not observed	86 ± 26	1120 ± 365
16b	**13a** (25%) + **13b** (20%)	210 ± 70	4500 ± 430

[a] Side-products that formed at the biochemical assay time-scale in solution and were identified by LC–MS.
[b] Values are means of 3 experiments.
[c] Starting from a single isomer after purification, the peptide oxidized readily in the buffer.

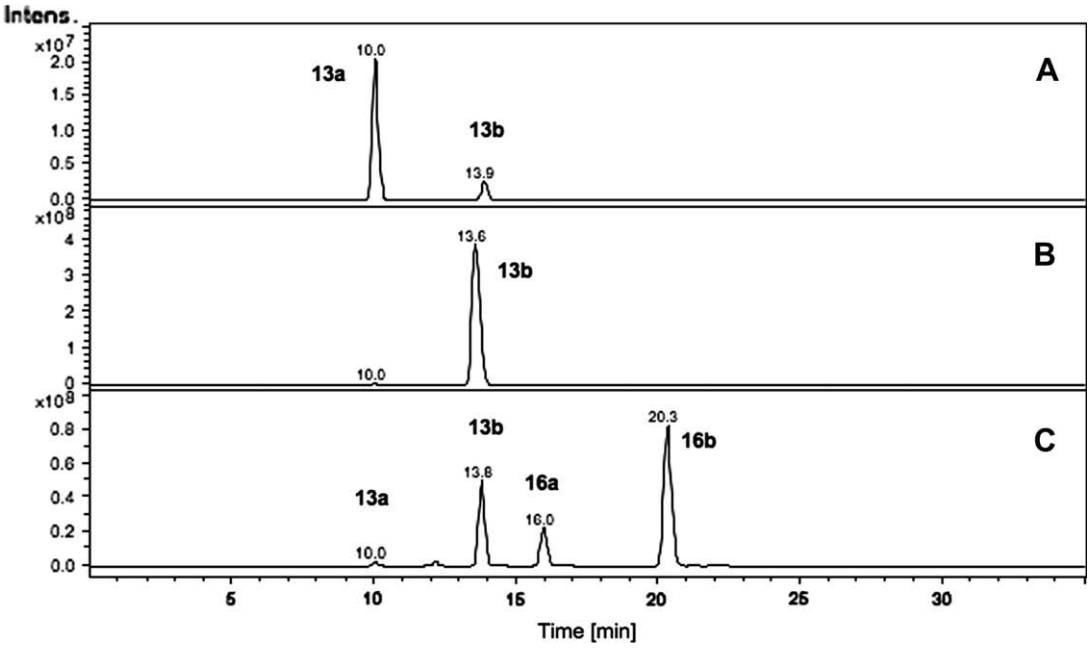


Fig. 3. HPLC chromatograms of initially pure compounds **13a** (A), **13b** (B) and **16b** (C) after storage at –20 °C in a 20 mM Tris HCl buffer pH 7.4.

37 °C for increasing periods of time, and aliquots were analyzed by LC–MS (data not shown). Although the proportion of complex **13b** did not vary significantly over 15 h, we observed that the ratio of **13a** decreases whereas ratios of **16a** and **b** slightly augment. These data are in agreement with the above mentioned results obtained with pure compounds and seem to confirm a putative interconversion of monomers and dimers. Moreover, the absence of detectable side-products resulting from complex dissociation clearly shows that these oxorhenium complexes are chemically stable over prolonged period of time and that the inhibition of integrin-echistatin interaction observed *in vitro* does not result from the dissociation of the oxorhenium complexes.

We investigated the sensitivity of compounds **12** and **13** to trypsin, a protease that hydrolyzes many peptides and peptide derivatives containing an arginine residue. For this purpose, we incubated compound **12** (either in its monomer or dimer form) with trypsin in a 35 mM HEPES buffer pH 8.6. As expected, LC–MS chromatograms showed that the R–G amide bond of the linear peptide was readily hydrolyzed at a 1% molar concentration in protease. Conversely, both isomers **13a** and **b** resisted up to a 10% molar concentration in trypsin. These results indicate that the oxorhenium complexes **13a** and **b** display a high resistance to trypsin-catalyzed hydrolysis. This result is likely to reflect a constrained conformation that mainly arises from a particular

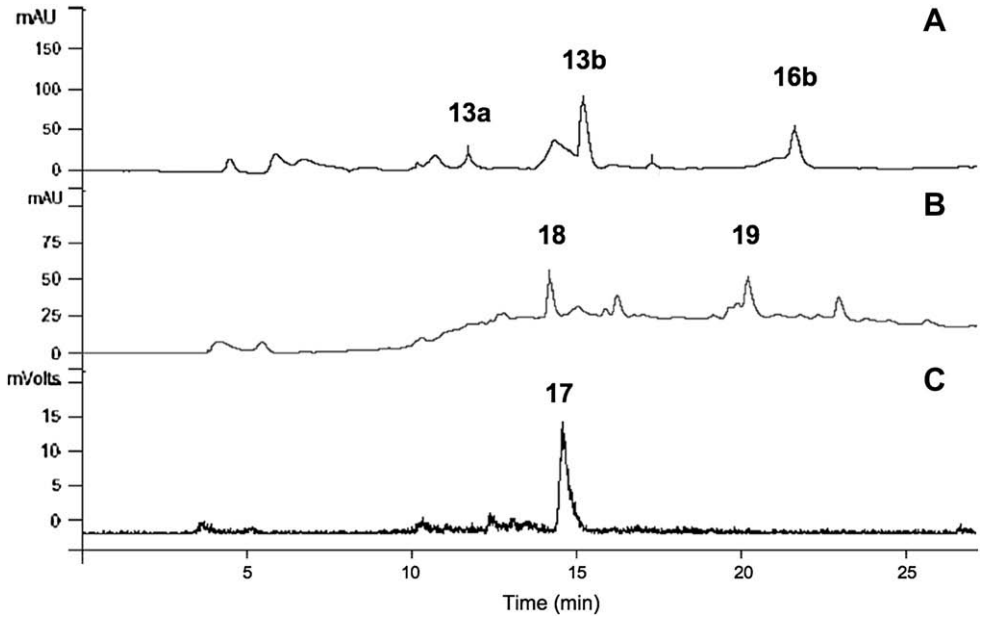


Fig. 4. HPLC chromatogram of compounds **13a**, **b** and **16b** (A) obtained through oxorhenium coordination of peptide **12** and radiochromatogram of the corresponding oxotechnetium complex (C) obtained as a single product. For comparison, peptide **12** metallated with $Bu_4N^{99g}TcOCl_4$ gave a mixture of monomer **18** and dimer **19** (B).

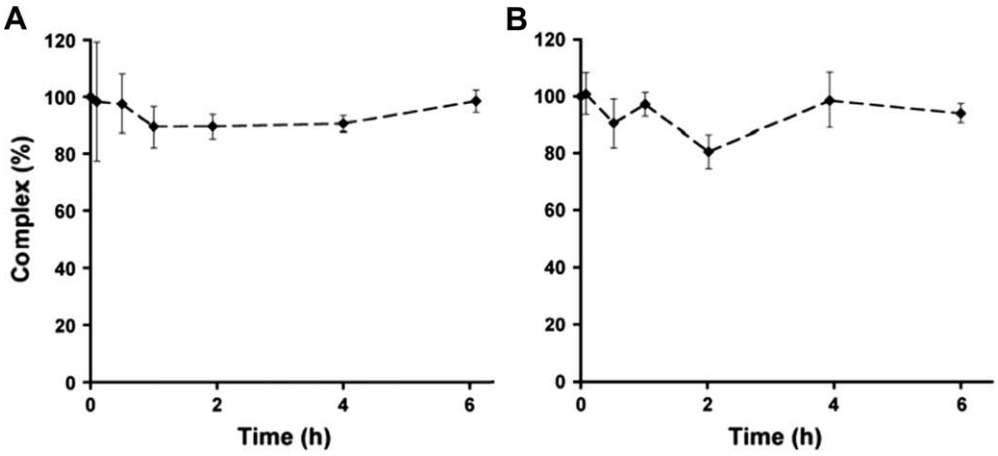


Fig. 5. Stability of complex **17** in the presence of 1 mM GSH in a 35 mM HEPES buffer pH 7.4 (A) and in mice plasma (B).

arrangement around the oxorhenium core. In contrast, the NS_2/S motif alone is not sufficient for protecting peptide **12** which is readily hydrolyzed by trypsin.

Labeling of peptide **12** with oxotechnetium using [^{99m}Tc]-oxotechnetium gluconate gave exclusively complex **17** (RP-HPLC $t_R = 14.2$ min) (Scheme 1 & Fig. 4C), the technetium equivalent of the oxorhenium complex **13b** (RP-HPLC $t_R = 15.2$ min) (Fig. 4A). This may be explained by the marked differences in kinetics of complexation between Tc and Re as well as distinct concentrations used for reaction with tetrabutylammonium tetrachlorooxorhenate (30 mM) and [^{99m}Tc]oxotechnetium gluconate (in the nanomolar range). This was confirmed by cyclization of peptide **12** with ^{99g}Tc, a long-lived β-emitter that may be handled at the milligram scale [48]. Home-made [^{99g}Tc]tetrabutylammonium tetrachlorooxotechnenate (40 mM in methanol) [54,55] gave a mixture of monomer **18** (RP-HPLC $t_R = 14.3$ min) and dimer **19** (RP-HPLC $t_R = 20.3$ min) (Fig. 4B).

Finally, we evaluated the resistance of complex **17** towards endogenous thiols such as glutathione (GSH) that might substitute the S moiety of the NS_2/S motif and therefore, could lead to the dissociation of the complex [57]. In the same way, prolonged contact with plasma proteins that contain free thiols such as serum albumin, might also dissociate complex **17**. We monitored the evolution of a solution of complex **17** in a 35 mM HEPES buffer pH 7.8 in the presence of 1 mM GSH on the one hand and in murine plasma on the other hand. As shown in Fig. 5, the tracer displayed a satisfactory stability towards excess of GSH (Fig. 5A) and in murine plasma (Fig. 5B) since no significant degradation of complex **17** could be detected after 6 h.

4. Conclusion

In conclusion, we designed and synthesized a RGD peptide **12** which was cyclized through oxorhenium coordination to give the cyclic metallated peptide **13**. Reaction with tetrabutylammonium tetrachlorooxorhenate gave a mixture of diastereomers **13a** and **b** and dimers **16a** and **b**. Compound **13b** was able to inhibit the binding of [^{125}I]echistatin to integrin $\alpha_V\beta_3$ with an IC_{50} which is equivalent to that of cyclopeptide c(RGDyV) **3**, a known antagonist of $\alpha_V\beta_3$. Rhenium complex **13b** exhibits a 13 times lower affinity for integrin $\alpha_{IIb}\beta_3$. Complex **13b** showed a satisfactory configurational stability at the experiment time scale, whereas slow but significant interconversions of isomer **13a** and dimer **16b** into **13b** were detected. This result suggest that their affinity for $\alpha_V\beta_3$ might be essentially related to the presence of **13b**, and that isomers **13a**, **16a** and **b** might possess a much lower activity than calculated from the competition assays. Reaction of peptide **12** with [^{99m}Tc]oxotechnetium gluconate gave exclusively the cyclic complex **17** which was identified as the equivalent of complex **13b**. This technetium conjugate showed a good stability towards GSH and plasma proteins. Further studies to investigate the potential of this compound as a tracer for the molecular imaging of integrin-dependent neoangiogenesis are under progress.

Acknowledgements

We thank the *Institut National du Cancer* (INCa) for financial support of this research program (Grant No. PL06-060). We gratefully acknowledge Dr. Carole Gaillard for iodination of echistatin and Bertrand Czarny for preparation of fresh mice plasma. We are indebted to Dr. Robert Thai and Steven Dubois for LC–MS analysis. We also express our thanks to Dr. Denis Servent for helpful advices and discussions.

References

[1] R.O. Hynes, Cell 110 (2002) 673–687.
[2] F.M. Watt, EMBO. J 21 (2002) 3919–3926.
[3] R. Haubner, D. Fisinger, H. Kessler, Angew. Chem. Int. Ed. Engl. 36 (1997) 1374–1389.
[4] G.J. Mizejewski, Proc. Soc. Exp. Biol. Med.. 222 (1999) 124–138.
[5] B. Wehrle-Haller, B.A. Imhof, J. Pathol. 200 (2003) 481–487.
[6] C.C. Kumar, H. Nie, C. Prorock Rogers, M. Malkowski, E. Maxwell, J. Catino, L.J. Armstrong, Pharmacol. Exp. Therapeut. 283 (1997) 843–853.
[7] J.D. Humphries, A. Byron, M.J. Humphries, J. Cell Sci. 119 (2006) 3901–3903.
[8] M.A. Dechantsreiter, E. Planker, B. Mathä, E. Lohof, G. Hölzemann, A. Jonczyk, S.L. Goodman, H. Kessler, J. Med. Chem. 42 (1999) 3033–3040.
[9] J.-P. Xiong, T. Stehle, R. Zhang, A. Joachimiak, M. Frech, S.L. Goodman, M.A. Arnaout, Science 296 (2002) 151–155.
[10] J.W. Smith, Curr. Opin. Investig. Drugs 4 (2003) 741–745.
[11] S.L. Goodman, G. Hölzemann, G.A. Sulyok, H. Kessler, J. Med. Chem. 45 (2002) 1045–1051.
[12] R. Haubner, H.-J. Wester, U. Reuning, R. Senekowitsch-Schmidtke, B. Diefenbach, H. Kessler, G. Stöcklin, M. Schwaiger, J. Nucl. Med. 40 (1999) 1061–1071.
[13] E. Lohof, E. Planker, C. Mang, F. Burkhart, M.A. Dechantsreiter, R. Haubner, H.J. Wester, M. Schwaiger, G. Hölzemann, S.L. Goodman, H. Kessler, Angew. Chem. Int. Ed. Engl. 39 (2000) 2761–2764.
[14] R.M. van Well, H.S. Overkleeft, G.A. van der marel, D. Bruss, G. Thibault, P.G. de Groot, J.H. van Boom, M. Overhand, Biorg. Med. Chem. Lett. 13 (2003) 331–334.
[15] L. Belvisi, A. Bernardi, A. Checchia, L. Manzoni, D. Potenza, C. Scolastico, M. Castorina, A. Cupelli, G. Giannini, P. Carminati, C. Pisano, Org. Lett. 3 (2001) 1001–1004.
[16] S. Urman, K. Gaus, Y. Yang, V. Strijowski, N. Sewald, S. De Pol, O. Reiser, Angew. Chem. Int. Ed. 46 (2007) 3976–3978.
[17] G. Casiraghi, G. Rassu, L. Auzzas, P. Burreddu, E. Gaetani, L. Battistini, F. Zanardi, C. Curti, G. Nicastro, L. Belvisi, I. Motto, M. Castorina, G. Giannini, C. Pisano, J. Med. Chem. 48 (2005) 7675–7687.

[18] F. Zanardi, P. Burreddu, G. Rassu, L. Auzzas, L. Battistini, C. Curti, A. Sartori, G. Nicastro, G. Menchi, N. Cini, A. Bottonocetti, S. Raspanti, G. Casiraghi, J. Med. Chem. 51 (2008) 1771–1782.
[19] A. Kelleman, H. R-Mattern, M.D. Pierschbacher, M. Goodman, Biopolymers 71 (2003) 686–695.
[20] B. Cacciari, G. Spalluto, Curr. Med. Chem. 12 (2005) 51–70.
[21] S.A. Moussa, Mol. Biotechnol. 38 (2008) 33–40.
[22] M. Albrecht, P. Stortz, J. Runsink, P. Weis, Chem. Eur. J. 10 (2004) 3657–3666.
[23] G.P. Curley, H. Blum, M.J. Humphries, Cell Mol. Life Sci. 56 (1999) 427–441.
[24] K. Temming, R.M. Schiffelers, G. Molema, R.J. Kok, Drug Resist. Updat. 8 (2005) 381–402.
[25] R. Haubner, H.-W. Wester, F. Burkhart, R. Senekowitsch-Schmidtke, W. Weber, S.L. Goodman, H. Kessler, M. Schwaiger, J. Nucl. Med. 42 (2001) 326–336.
[26] R. Haubner, W. H-Wester, W.A. Weber, C. Mang, S.I. Ziegler, S.L. Goodman, R. Senekowitsch-Schmidtke, H. Kessler, M. Schwaiger, Cancer Res. 61 (2001) 1781–1785.
[27] M.L. Janssen, W.J. Oyen, I. Dijkgraaf, L.F. Massuger, C. Frielink, D.S. Edwards, M. Rajopadhye, H. Boonstra, F.H. Korstens, O. Boerman, Cancer Res. 62 (2002) 6146–6151.
[28] X. Chen, M. Tohme, R. Park, Y. Hou, J.R. Bading, P.S. Conti, Mol. Imaging 3 (2004) 96–104.
[29] X. Chen, R. Park, A.H. Shahinian, J.R. Bading, P.S. Conti, Nucl. Med. Biol. 31 (2004) 11–19.
[30] X. Chen, R. Park, A.H. Shahinian, M. Tohme, V. Klankaldyyan, M.H. Bozorgzadeh, J.R. Bading, R. Moats, W.E. laug, P.S. Conti, Nucl. Med. Biol. 31 (2004) 179–189.
[31] X. Chen, R. Park, Y. Hou, V. Klankaldyyan, I. Gonzales-Gomez, M. Tohme, J.R. Bading, W.E. Laug, P.S. Conti, Eur. J. Nucl. Med. Mol. Imaging 31 (2004) 1081–1089.
[32] X. Chen, R. Park, Y. Hou, V. Klankaldyyan, I. Gonzales-Gomez, M. Tohme, J.R. Bading, W.E. Laug, P.S. Conti, J. Nucl. Med. 45 (2004) 1776–1783.
[33] R. Haubner, F. Bruchertseifer, M. Bock, H. Kessler, M. Schwaiger, H.-J. Wester, Nuklearmedizin 1 (2004) 26–32.
[34] X. Chen, R. Park, M. Tohme, A.H. Shahinian, J.R. Bading, P.S. Conti, Bioconjug. Chem. 15 (2004) 41–49.
[35] R. Haubner, B. Kuhnast, C. Mang, W.A. Weber, H. Kessler, W. H-Wester, M. Schwaiger, Bioconjug. Chem. 15 (2004) 61–69.
[36] Z. Cheng, Y. Wu, Z. Xiong, S.S. Gambhir, X. Chen, Bioconjug. Chem. 16 (2005) 1433–1441.
[37] Y. Wu, X. Zhang, Z. Xiong, Z. Cheng, D.R. Fischer, S. Liu, S. Gambhir, X. Chen, J. Nucl. Med. 46 (2005) 1707–1718.
[38] X. Zhang, Z. Xiong, Y. Wu, W. Cai, J.R. Tseng, S.S. Gambhir, X. Chen, J. Nucl. Med. 47 (2006) 113–121.
[39] L. Sancey, V. Ardisson, L.M. Riou, M. Ahmadi, D. Martibattle, D. Boturyn, P. Dumy, D. Fagret, C. Ghezzi, J.P. Vuillez, Eur. J. Nucl. Med. Mol. Imaging 34 (2007) 2037–2047.
[40] Z.-H. Jin, J. Razkin, V. Josserand, D. Boturyn, A. Grichine, I. Texier, M.-C. Favrot, P. Dumy, J.-L. Coll, Mol. Imaging 6 (2007) 43–55.
[41] Z.-H. Jin, J. Josserand, S. Foillard, D. Boturyn, P. Dumy, M.-C. Favrot, J.-L. Coll, Mol. Cancer 6 (2007) 41.
[42] J. Dimastromatteo, G. Pons, L. Riou, E. Pellegrini, T. Gavrilina, M. Ahmadi, D. Boturyn, P. Dumy, D. Fagret, C. Ghezzi, Circulation 116 (2008) 659–660.
[43] M. Albrecht, P. Stortz, Chem. Soc. Rev. 34 (2005) 496–506.
[44] Y. Barda, N. Cohen, V. Lev, N. Ben-Aroya, Y. Koch, E. Mishani, M. Fridkin, C. Gilon, Nucl. Med. Biol. 31 (2004) 921–933.
[45] G. Fridkin, T.A. Bonasera, P. Litman, C. Gilon, Nucl. Med. Biol. 32 (2005) 39–50.
[46] L. Wei, C. Butcher, Y. Miao, F. Gallazzi, T.P. Quinn, M.J. Welch, J.S. Lewis, J. Nucl. Med. 48 (2007) 64–72.
[47] H.M. Bigott-Hennkens, S. Junnotula, L. Ma, F. Gallazzi, M.R. Lewis, S.S. Jurisson, J. Med. Chem. 51 (2008) 1223–1230.
[48] J.R. Dilworth, S.J. Parrott, Chem. Soc. Rev. 27 (1998) 43–55.
[49] S. Liu, S. Edwards, Chem. Rev. 99 (1999) 2235–2268.
[50] C. Clavaud, M. Heckenroth, C. Stricane, A. Ménez, C. Dugave, Bioconjug. Chem. 17 (2006) 807–814.
[51] M.S. Papadopoulos, I.C. Pirmettis, M. Pelecanou, C.P. Raptopoulou, A. Terzis, C.I. Stassinopoulou, E. Chiotellis, Inorg. Chem. 35 (1996) 7377–7383.
[52] M. Pelecanou, K. Chryssou, C.I. Stassinopoulou, J. Inorg. Biochem. 79 (2000) 347–351.
[53] A. Davison, C. Orvig, H.S. Trop, M. Sohn, B.V. DePamphilis, Inorg. Chem. 19 (1980) 1988–1992.
[54] A. Davison, H. Trop, B. DePamphilis, A. Jones, Inorg. Synth. 21 (1982) 160–162.
[55] S. Friedrich-Bochnitschek, H. Waldmann, H. Kunz, J. Org. Chem. 54 (1989) 751–756.
[56] R.A. Orlando, D.A. Cheresh, J. Biol. Chem. 266 (1991) 19543–19550.
[57] C. Clavaud, J. Le Gal, R. Thai, M. Moutiez, Chem. Biol. Chem. 9 (2008) 1823–1829.