Functionalization of Ruthenium(II) Terpyridine Complexes with Cyclic RGD Peptides To Target Integrin Receptors in Cancer Cells

Hahn, Eva M.; Estrada Ortiz, Natalia; Han, Jiaying; Ferreira, Vera F. C.; Kapp, Tobias G.; Correia, Joao D. G.; Casini, Angela; Kuehn, Fritz E.

Published in:
European Journal of Inorganic Chemistry

DOI:
10.1002/ejic.201601094

Publication date:
2017

ChemPubSoc Europe

DOI: 10.1002/ejic.201601094

EurJIC European Journal of Inorganic Chemistry

Communication

Targeting Integrin Receptors

CLUSTER ISSUE

Functionalization of Ruthenium(II) Terpyridine Complexes with Cyclic RGD Peptides To Target Integrin Receptors in Cancer Cells

Eva M. Hahn,[a,b] Natalia Estrada-Ortiz,[c] Jiaying Han,[c] Vera F. C. Ferreira,[d] Tobias G. Kapp,[e] João D. G. Correia,[d] Angela Casini,*[b,c,e] and Fritz E. Kühn*[a]

Abstract: The lack of selectivity for cancer cells and the resulting negative impact on healthy tissue is a severe drawback of actual cancer chemotherapy. Tethering of cytotoxic drugs to targeting vectors such as peptides, which recognize receptors overexpressed on the surface of tumor cells, is one possible strategy to overcome such a problem. The pentapeptide cyc(RGDfK) targets the integrin receptor $\alpha_v\beta_3$, important for tumor growth and metastasis formation. In this work, two terpyridine-based Ru^{II} complexes were prepared and for the first time conjugated to cyc(RGDfK) through amide bond formation, which resulted in a monomeric and a dimeric bioconjugate. Both Ru^{II} complexes were found to bind strongly and selectively to integrin $\alpha_v\beta_3$, and the dimeric molecule displayed a 20-fold higher affinity to the receptor than the monomeric one. However, the cytotoxicity of the complexes and related bioconjugates against human A549 and SKOV-3 cell lines is still not sufficient for application as anticancer agents. Nevertheless, considering the high selectivity for integrin receptor $\alpha_v\beta_3$, the synthesis of Ru-based bioconjugates with cyc(RGDfK) paves a promising way towards the design of effective targeted anticancer agents.

Introduction

Platinum anticancer drugs are widely used for chemotherapy of various cancers. However, indiscriminate distribution or poor selectivity often results in severe side effects and drug resistance.[1] Therefore, enhancing the tumor selectivity has become a major goal for the development of platinum-based cytotoxic agents. Similar issues are encountered with the new generation of experimental anticancer metal complexes, including, among others, compounds based on ruthenium,[2] gold,[3] iron,[4] and copper.[5] Thus, the development of so-called targeting and drug-delivery strategies of metallodrugs has become a priority in the field, together with the design of new chemical scaffolds.

Within this framework, an increasing number of reports on tethering metal complexes to a wide range of functional molecules or nanoparticles with or without targeting groups has appeared in recent years.[6] Specifically, the functionalization of metallodrugs is aimed at improving tumor selectivity and/or minimizing systemic toxicity to enhance their cellular accumulation and to overcome tumor resistance. Moreover, a synergistic anticancer effect of different therapeutic modalities would also be welcome. In some cases, the use of imaging tags conjugated to the metal compounds allows visualization of the drug molecules in vitro or in vivo, which thus leads to the design of theranostic agents.[7]

Among the various strategies explored so far to actively target cytotoxic metallodrugs to cancer cells, tumor-targeting peptides (TTPs), which are specific for tumor-related surface markers, such as membrane receptors, can be used.[8]

Integrin receptors have been largely explored as drug targets, as they are heterodimeric, transmembrane receptors that function as mechanosensors, adhesion molecules, and signal transduction platforms in a multitude of biological processes.[9] Integrins interact with the extracellular matrix (ECM) and thereby regulate many cellular functions, such as proliferation, migration, and survival. Integrins are also involved in cell-to-cell interactions. Through cell–cell and cell–ECM contacts, integrins transduce the information from the external environment into the cell and vice versa to promote cell adhesion, spreading, and motility.[10] One common feature of the integrin family is a heterodimeric structure that consists of α and β subunits.[11] These structures form 24 different subtypes in mammals, which can be classified according to their binding partners (e.g. laminin, collagen). Different integrins are also associated with tumor angiogenesis and metastasis,[12] which are upregulated in tumor cells relative to the low levels in normal endothelial

[a] *Molecular Catalysis, Catalysis Research Center and Department of Chemistry, Technische Universität München, Lichtenbergstr. 4, 85747 Garching bei München, Germany E-mail: fritz.kuehn@ch.tum.de*

[b] *School of Chemistry, Cardiff University, Park Place, Cardiff CF103AT, United Kingdom E-mail: casinia@cardiff.ac.uk*

[c] *Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands*

[d] *Centro de Ciências e Tecnologias Nucleares, Instituto Superior Técnico, Universidade de Lisboa, CTN, Estrada Nacional 10 (km 139.7), 2695-066 Bobadela LRS, Portugal*

[e] *Institute for Advanced Study, Technische Universität München, Lichtenbergstr. 2a, 85748 Garching, Germany*

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejic.201601094.

cells. The integrin receptor $\alpha_v\beta_3$ plays a crucial role in these processes[13,14] and has become an attractive target for pharmaceutical research.[15]

In 1984, Pierschbacher and Ruoslahti discovered that the amino acid sequence Arg-Gly-Asp-Ser (RGDS) is essential for binding integrin receptors.[16] In fact, eight of the abovementioned integrin subtypes form the RGD-binding class.[17] Since then, a wide screening of peptide libraries has been performed to discover ligands including the RGD sequence and targeting integrin receptors with even higher selectivity. Interestingly, the cyclic pentapeptide cyc[RGDfK] (Figure 1) was found to have increased selectivity for integrin $\alpha_v\beta_3$.[18]

Among the metal-based radiopharmaceuticals tethered to cyclic RGD peptides, the majority of the reported examples were evaluated as single-photon emission computed tomography (SPECT) and positron emission tomography (PET) radiotracers for tumor imaging.[7a,21] Recently, preclinical evaluation of the potential theranostic radiopharmaceutical ^{66}Ga-DOTA-E(cyc[RGDfK])$_2$ compound was reported.[22]

As an example of targeted anticancer metal complexes, recent reports describe the synthesis and biological evaluation of Pt^{IV} prodrugs, the axial positions of which could be functionalized with cyclic RGD tripeptides that bind selectively to the integrin receptor $\alpha_v\beta_3$.[19,23] In a more elaborated approach, Lippard et al. synthesized a cisplatin prodrug encapsulated into poly(D,L-lactic-co-glycolic acid)-block-polyethylene glycol (PLGA-PEG) nanoparticles tethered to cyc[RGDfK]. The prodrug shows a significant increase in cytotoxicity towards $\alpha_v\beta_3$ integrin-expressing cancer cell lines, comparable to cisplatin. In vivo studies also revealed equivalent tumor growth inhibition (ca. 60 %) by both the prodrug and cisplatin in mice bearing ovarian cancer xenografts.[20]

Concerning anticancer ruthenium complexes coupled to peptides, some examples have already been reported,[8] including luminescent Ru^{II} complexes linked through the mitochondrial penetrating peptide (MPP)[24] as well as to the nuclear localization sequence (NLS),[25] the latter of which enables the active transport of drugs into the cell nucleus as confirmed by fluorescence microscopy studies. Interestingly, Keyes et al. developed ruthenium(II) polypyridyl luminophores anchored to peptide sequences as a new class of stimulated emission depletion (STED) microscopy probes for the imaging of key cell organelles.[26] Ueyama et al. also described a peptide-labeling approach by using Ru^{II} terpyridine complexes to implement the mass spectrometry detection of proteolytic peptides.[27]

As far as it concerns RGD-type peptides, only a few examples are described. Thus, Sadler et al. reported the synthesis of a Ru^{II} arene complex attached to the linear RGD tripeptide[28] that dissociated from the peptide by irradiation with visible light to form an aqua complex that generated monofunctional adducts with the guanine bases of DNA. Furthermore, Adamson et al. designed luminescent Ru^{II} polypyridyl complexes attached to the linear RGD tripeptide, which acted as molecular probes for reporting the presence and conformation of integrins.[29] Live-cell studies with confocal microscopy confirmed the selective binding to an integrin receptor, but no cytotoxicity studies were described.

Finally, fluorescent ruthenium polypyridyl complexes were attached to RGD-functionalized mesoporous silica nanoparticles,[30] the uptake and subcellular distribution of which could be followed by fluorescence microscopy. Interestingly, the RDG peptide on the nanoparticle surface induced an increased selectivity for cancer cells. After internalization of the nanoparticle, the ruthenium species were released and induced changes

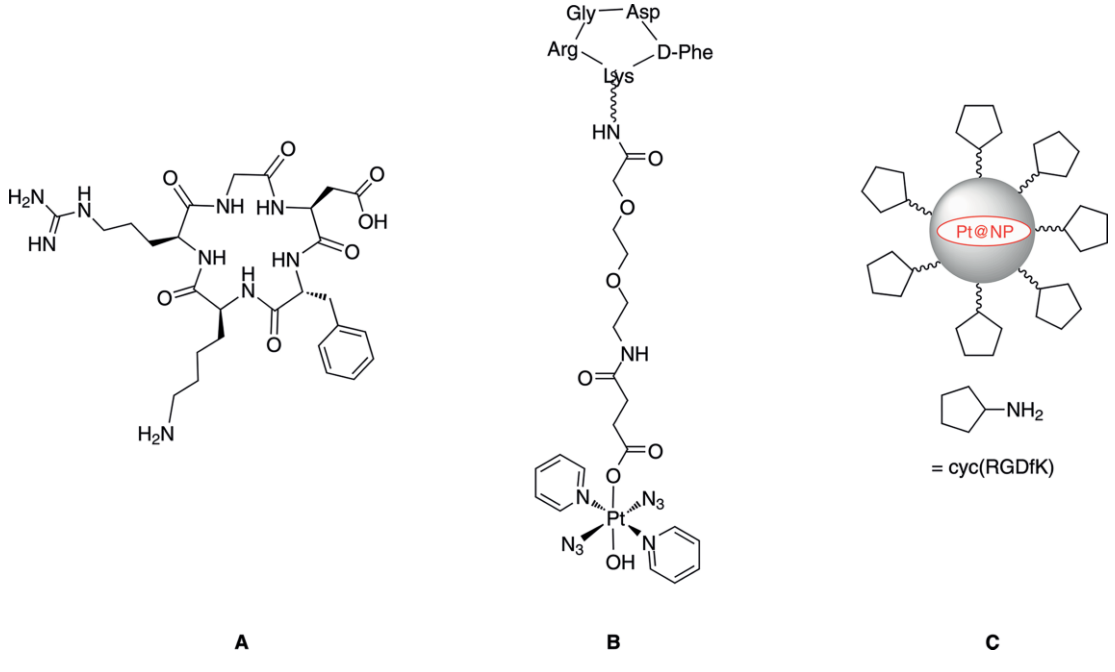


Figure 1. Cyclic pentapeptide cyc[RGDfK] (**A**) and two representative targeted Pt constructs: the Pt^{IV} conjugate[19] (**B**) and nanoparticles encapsulating a cisplatin prodrug[20] (**C**).

in the phosphorylation pathway of certain protein kinases, which led to apoptosis.

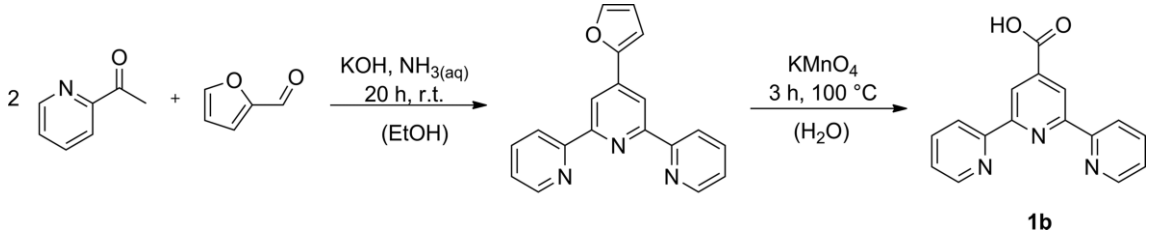
To the best of our knowledge, besides the abovementioned publications, there are no other studies about the conjugation of ruthenium complexes to RGD-type peptides. Therefore, in this work the successful bioconjugation of two Ru^{II} terpyridine complexes to the cyclic RGD peptide cyc[RGDfK] for targeting integrin $\alpha_v\beta_3$ is reported. The two Ru^{II} compounds were designed to feature carboxylic acid groups for conjugation to the lysine residue of the RGD peptide through amide bond formation. Thus, the compounds were tethered to one or two peptides. In the latter case, anchoring to two cyc[RGDfK] was intended to enhance the binding affinity to the $\alpha_v\beta_3$ integrin receptor.[13] The binding affinities of the ruthenium–RGD conjugates for both the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin receptors were evaluated by integrin binding assays. The anticancer effects of the "free" ruthenium complexes and their respective conjugates were evaluated in vitro against human cancer cell lines with different expression levels of integrin $\alpha_v\beta_3$, namely, human lung cancer A549 cells (scarce $\alpha_v\beta_3$ integrin expression) and human mammary carcinoma SKOV3 cells (moderate $\alpha_v\beta_3$ integrin expression).[31]

Results and Discussion

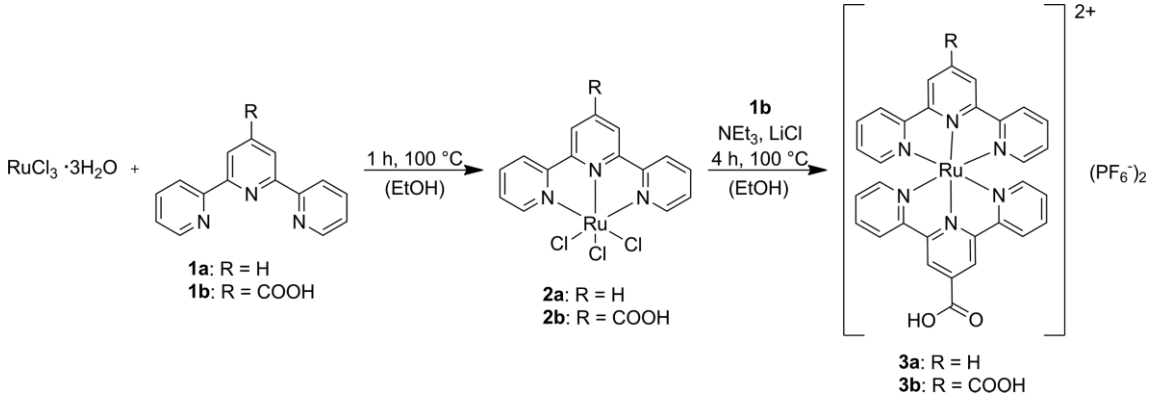
The experimental procedures can be found in the Supporting Information. The two ligands used in this work are 2,2′:6′,2″-terpyridine (terpy, **1a**) and [2,2′:6′,2″-terpyridine]-4′-carboxylic acid (terpy*, **1b**). For the synthesis of **1b**, a reported two-step procedure was followed.[32] In the first step, 2-acetylpyridine and furfural were combined in ethanol under basic conditions to yield 4′-(furan-2-yl)terpyridine, which was oxidized in the following step with $KMnO_4$ to obtain [2,2′:6′,2″-terpyridine]-4′-carboxylic acid (terpy*, **1b**) (see Scheme 1).

Complexes **3a** and **3b** were prepared by a novel synthetic route based on literature procedures[33] (Scheme 2). Heating $RuCl_3 \cdot 3H_2O$ with **1a** or **1b** in dry ethanol yielded brown complex **2a** or **2b**, respectively, after 1 h in the dark. Afterwards, the complexes were separately treated with **1b**, triethylamine, and LiCl for chloride abstraction and reduction of Ru^{III} to Ru^{II}. Upon the addition of 1 M KPF_6, the $[Ru(terpy)(terpy^*)](PF_6)_2$ (**3a**) and $[Ru(terpy^*)_2](PF_6)_2$ (**3b**) complexes bearing one and two carboxylic acid groups, respectively, precipitated.

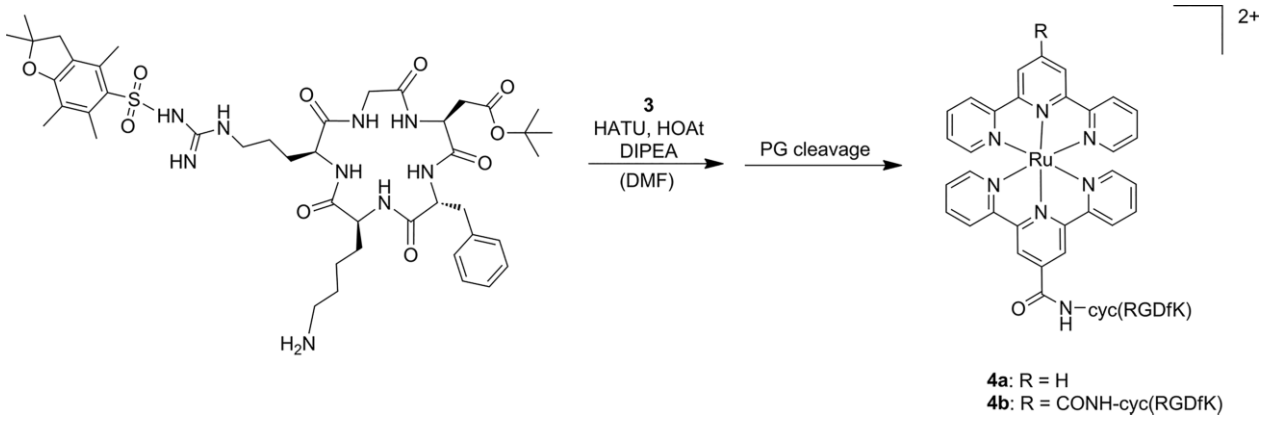
Conjugation of **3a** and **3b** to the cyclic peptide cyc[R(Pbf)GD(*t*Bu)fK] was accomplished by reaction of the free carboxylic acid groups of the complexes with the primary amine group of the lysine side chain in the presence of a mixture of the activating agents 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt) (Scheme 3). The success of the bioconjugation reaction was confirmed by electrospray ionization mass spectrometry (ESI-MS), which allowed identification of the intermediate products at m/z = 752.78 for $[Ru(terpy)\{terpy\text{-}cyc\{R(Pbf)GD(tBu)fK\}\}]^{2+}$ and 1221.58 for $[Ru\{terpy\text{-}cyc\{R(Pbf)GD(tBu)fK\}\}_2]^{2+}$, respectively. Afterwards, the remaining protecting groups of Arg and Asp were cleaved by using a cleavage cocktail as detailed in the Experimental Section. For purification of the crude product, size-exclusion chromatography with Sephadex G-15 was used, as the compounds decomposed during reverse-phase (RP)-HPLC. Finally, the products were precipitated by the addition of solid KPF_6 to give $[Ru(terpy)\{terpy\text{-}cyc(RGDfK)\}](PF_6)_2$ (**4a**) and $[Ru\{terpy\text{-}cyc(RGDfK)\}_2](PF_6)_2$ (**4b**) as red solids.



Scheme 1. Synthesis of the [2,2′:6′,2″-terpyridine]-4′-carboxylic acid ligand (**1b**).



Scheme 2. Two-step procedure for the synthesis of $[Ru(terpy)(terpy^*)](PF_6)_2$ (**3a**) and $[Ru(terpy^*)_2](PF_6)_2$ (**3b**).



Scheme 3. Synthesis of bioconjugate products **4a** and **4b** (PG = protecting group; DIPEA = *N*,*N*-diisopropylethylamine).

Characterization of Ligand 1b Complexes 3a/3b and 4a/4b

The ligands and the corresponding complexes were characterized by ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectroscopy and ESI-MS.

Comparing the ^{1}H NMR spectra of ligand **1b** with **3a**, several signal shifts are observed owing to complex formation (Figure 2). The signals of $H^{3',5'}$ and $H^{3,3''}$ are shifted downfield by around $\Delta\delta$ = +0.61 and +0.49 ppm. In contrast, the signal of $H^{4,4''}$ remains, and the signals of $H^{6,6''}$ and $H^{5,5''}$ show a strong upfield shift of $\Delta\delta$ = –1.25 and –0.28 ppm. Nearly the same values are observed for complex **3b** containing two ligands **1b**. The downfield shifts of $H^{3',5'}$ and $H^{3,3''}$ are about $\Delta\delta$ = +0.62 and +0.47 ppm, whereas the signal of $H^{4,4''}$ remains, and the signals of $H^{6,6''}$ and $H^{5,5''}$ are shifted upfield by about $\Delta\delta$ = –1.20 and –0.26 ppm. For these observations, two effects have to be taken into account: first, the deshielding effect of the carboxylic acid group; second, the increase in electron density in the aromatic system through coordination of ruthenium. The remaining signals in the spectrum of **3a** can be assigned to coordinated ligand **1a**. In the ^{31}P NMR spectra, the presence of the PF_6^- counterions in complexes **3a** and **3b** is confirmed by the characteristic septet.

Complexes **3a** and **3b** and their conjugation derivatives **4a** and **4b** were characterized by ESI-MS, and the characteristic isotopic patterns are consistent with the assigned structures (Figures S3–S14 in the Supporting Information). The ESI mass spectra of the complexes show signals at *m*/*z* = 757.05 and 306.04 for **3a** and at *m*/*z* = 801.04 and 328.04 for **3b**, which indicate the loss of one or two PF_6^- anions, and this leads to a singly or doubly positive charged cationic species. Similarly, for coupling products **4a** and **4b**, the loss of the PF_6^- anions is observed. The characteristic isotopic patterns of the signals match perfectly with the calculated ones, which can be seen in the Supporting Information.

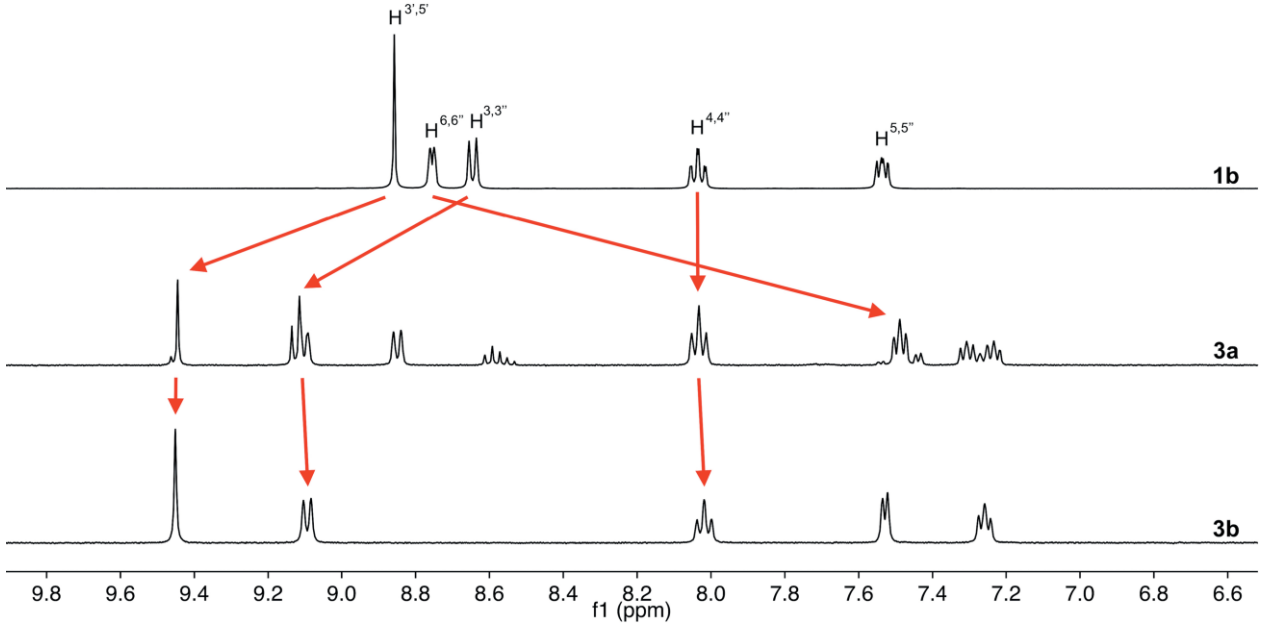


Figure 2. ^{1}H NMR spectra of **1b** and complexes **3a** and **3b** (in [D_6]DMSO).

Integrin Binding Assay

The impact of conjugation of the Ru^{II} complexes to cyc[RGDfK] on the binding affinity to the integrin receptors $\alpha_v\beta_3$ and $\alpha_5\beta_1$ was evaluated. The binding affinities for **4a**, **4b**, and benchmark Cilengitide[34] are shown in Table 1.

Table 1. Results of integrin binding assays for bioconjugates **4a** and **4b** in comparison to the benchmark Cilengitide.[a]

Compound	IC_{50} [nM] ± SD	
	$\alpha_v\beta_3$	$\alpha_5\beta_1$
Cilengitide[34]	0.54 ± 0.06	15.4 ± 0.2
4a	49 ± 4.3	>1000
4b	2.5 ± 0.3	595 ± 67

[a] The reported IC_{50} values were determined by using a solid-phase binding assay (see the Supporting Information for details).

Bioconjugate **4a** exhibits a median inhibitory concentration (IC_{50}) value of (49 ± 4.3) nM, which is 90-fold higher than that of Cilengitide [(0.54 ± 0.06) nM]. However, the selectivity for $\alpha_v\beta_3$ is reasonably high, which reflects the fact that the bioconjugate does not bind the $\alpha_5\beta_1$ receptor at all (IC_{50} > 1000 nM), whereas Cilengitide still has an affinity of (15.4 ± 0.2) nM. Considering bioconjugate **4b**, enhanced binding affinities are predicted owing to its dimeric character. Indeed, the binding affinity for integrin $\alpha_v\beta_3$ is (2.5 ± 0.3) nM, and it presents an affinity that is 20 times higher than that of the monomeric product and nearly approaches the value of Cilengitide. Given that the affinity for the $\alpha_5\beta_1$ receptor shows merely a value of about (595 ± 67) nM, the high selectivity of **4b** for $\alpha_v\beta_3$ is demonstrated.

Antiproliferative Activity

Ruthenium compounds **3a** and **3b** and respective cyc[RGDfK] bioconjugates **4a** and **4b** were evaluated for their antiproliferative properties on two human cancer cell lines with scarce (A549) or moderate (SKOV3) expression of integrin $\alpha_v\beta_3$.[31] Unfortunately, both ruthenium(II) complexes and their targeted derivatives showed similarly very low cytotoxic effects against both cell lines, independent of the presence of the RGD domains (Table 2). This could be attributed to the intrinsic limited anticancer effects of the selected Ru^{II} derivatives. Therefore, although their cell uptake should be favored by the presence of cyc[RGDfK] domains, in the end, no toxic effects were observed.

Table 2. IC_{50} values of Ru complexes and their RGD bioconjugates against human A549 and SKOV-3 cell lines.

Compound	IC_{50}[a] [μM]	
	A549	SKOV-3
3a	70.3 ± 9.8	74.5 ± 13.7
4a	87.7 ± 5.4	85.2 ± 18.7
3b	>100	>100
4b	>100	>100

[a] The reported values are the mean ± SD of at least three determinations.

Conclusions

In summary, two novel ruthenium(II) polypyridyl complexes coupled to the cyclic pentapeptide cyc[RGDfK] with monomeric or dimeric character were prepared to deliver anticancer metallodrugs directly to tumors cells overexpressing the $\alpha_v\beta_3$ integrin receptor.

The preparation of terpy-based ruthenium complexes **3a** and **3b** bearing one and two carboxylic acid groups, respectively, was performed by using a novel synthetic strategy. The compounds were coupled to a protected derivative of the cyclic pentapeptide through amide bond formation between the carboxylic acid of the complex and the amine group of the lysine side chain. Purification of resulting monomeric bioconjugate **4a** and dimeric bioconjugate **4b** was achieved by size-exclusion chromatography followed by precipitation as their PF_6 salts. Considering the binding affinities of the bioconjugates towards the integrin receptors, a high selectivity for the $\alpha_v\beta_3$ integrin receptor and a negligible impact on the $\alpha_5\beta_1$ receptor was observed. Still, the cytotoxicity of all the reported bioconjugates was low, most likely as a result of still-scarce uptake in cancer cells. Hence, whereas the reported strategy holds promise to achieve targeted metallodrugs, future studies have to focus on tethering to the RGD peptide of ruthenium complexes with an intrinsically higher cytotoxic potency, such as similar types of ruthenium complexes with terpyridine-type ligands.[35]

Acknowledgments

A.C. acknowledges support from Cardiff University, the August-Wilhelm Scheer Visiting Professorship at the Technical University of Munich (TUM), and a Hans Fischer Senior Fellowship of TUM IAS (Institute for Advanced Study) funded by the German Excellence Initiative and the European Union Seventh Framework Program under grant agreement n° 291763. E. M. H. acknowledges Prof. H. Kessler for helpful discussions and is grateful for financial support of the TUM Graduate School of Chemistry and the Short Term Scientific Mission supported by COST action CM1105. V. F. C. F. and J. D. G. C. gratefully acknowledge the FCT (Portugal) support through the UID/Multi/04349/2013 project.

Keywords: Antitumor agents · Bioconjugation · Peptides · Receptors · Ruthenium

[1] Y. Jung, S. J. Lippard, *Chem. Rev.* **2007**, *107*, 1387–1407.
[2] a) A. Bergamo, C. Gaiddon, J. H. M. Schellens, J. H. Beijnen, G. Sava, *J. Inorg. Biochem.* **2012**, *106*, 90–99; b) E. Meggers, *Chem. Commun.* **2009**, 1001–1010.
[3] B. Bertrand, A. Casini, *Dalton Trans.* **2014**, *43*, 4209–4219.
[4] G. Jaouen, A. Vessieres, S. Top, *Chem. Soc. Rev.* **2015**, *44*, 8802–8817.
[5] A. Rivero-Müller, A. De Vizcaya-Ruiz, N. Plant, L. Ruiz, M. Dobrota, *Chemico-Biological Interactions* **2007**, *165*, 189–199.
[6] a) X. Wang, X. Wang, Z. Guo, *Acc. Chem. Res.* **2015**, *48*, 2622–2631; b) J. S. Butler, P. J. Sadler, *Curr. Opin. Chem. Biol.* **2013**, *17*, 175–188.
[7] a) J. D. G. Correia, A. Paulo, P. D. Raposinho, I. Santos, *Dalton Trans.* **2011**, *40*, 6144–6167; b) R. Kumar, W. S. Shin, K. Sunwoo, W. Y. Kim, S. Koo, S. Bhuniya, J. S. Kim, *Chem. Soc. Rev.* **2015**, *44*, 6670–6683.
[8] M. Soler, L. Feliu, M. Planas, X. Ribas, M. Costas, *Dalton Trans.* **2016**, *45*, 12970–12982.
[9] a) M. Millard, S. Odde, N. Neamati, *Theranostics* **2011**, *1*, 154–188; b) S. J. Shattil, C. Kim, M. H. Ginsberg, *Nature Rev. Mol. Cell Biol.* **2010**, *11*, 288–300.
[10] K. R. Legate, S. A. Wickström, R. Fässler, *Genes Dev.* **2009**, *23*, 397–418.

[11] M. J. Humphries, *Biochem. Soc. Trans.* **2000**, *28*, 311–340.
[12] J. Xiong, H. E. Balcioglu, E. H. J. Danen, *Int. J. Biochem. Cell Biol.* **2013**, *45*, 1012–1015.
[13] M. Schottelius, B. Laufer, H. Kessler, H.-J. Wester, *Acc. Chem. Res.* **2009**, *42*, 969–980.
[14] R. J. Boohaker, M. W. Lee, P. Vishnubhotla, J. L. M. Perez, A. R. Khaled, *Curr. Med. Chem.* **2012**, *19*, 3794–3804.
[15] T. G. Kapp, F. Rechenmacher, T. R. Sobahi, H. Kessler, *Expert Opin. Ther. Pat.* **2013**, *23*, 1273–1295.
[16] M. D. Pierschbacher, E. Ruoslahti, *Nature* **1984**, *309*, 30–33.
[17] R. O. Hynes, *Cell* **2002**, *110*, 673–687.
[18] a) R. Haubner, D. Finsinger, H. Kessler, *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1374–1389; *Angew. Chem.* **1997**, *109*, 1440–1456; b) R. Haubner, R. Gratias, B. Diefenbach, S. L. Goodman, A. Jonczyk, H. Kessler, *J. Am. Chem. Soc.* **1996**, *118*, 7461–7472.
[19] A. Gandioso, E. Shaili, A. Massaguer, G. Artigas, A. Gonzalez-Canto, J. A. Woods, P. J. Sadler, V. Marchan, *Chem. Commun.* **2015**, *51*, 9169–9172.
[20] N. Graf, D. R. Bielenberg, N. Kolishetti, C. Muus, J. Banyard, O. C. Farokhzad, S. J. Lippard, *ACS Nano* **2012**, *6*, 4530–4539.
[21] a) J. Shi, L. Wang, Y.-S. Kim, S. Zhai, Z. Liu, X. Chen, S. Liu, *J. Med. Chem.* **2008**, *51*, 7980–7990; b) J. Shi, F. Wang, S. Liu, *Biophysics Rep.* **2016**, *2*, 1–20.
[22] V. Lopez-Rodriguez, R. E. Gaspar-Carcamo, M. Pedraza-Lopez, E. L. Rojas-Calderon, C. Arteaga de Murphy, G. Ferro-Flores, M. A. Avila-Rodriguez, *Nucl. Med. Biol.* **2015**, *42*, 109–114.
[23] S. Mukhopadhyay, C. M. Barnés, A. Haskel, S. M. Short, K. R. Barnes, S. J. Lippard, *Bioconjugate Chem.* **2008**, *19*, 39–49.
[24] A. Martin, A. Byrne, C. S. Burke, R. J. Forster, T. E. Keyes, *J. Am. Chem. Soc.* **2014**, *136*, 15300–15309.
[25] L. Blackmore, R. Moriarty, C. Dolan, K. Adamson, R. J. Forster, M. Devocelle, T. E. Keyes, *Chem. Commun.* **2013**, *49*, 2658–2660.
[26] A. Byrne, C. S. Burke, T. E. Keyes, *Chem. Sci.* **2016**, *7*, 6551–6562.
[27] A. Ito, T. A. Okamura, K. Masui, M. Kaneko, R. Masui, K. Ake, S. Kuramitsu, M. Yamaguchi, H. Kuyama, E. Ando, S. Norioka, T. Nakazawa, S. Tsunasawa, H. Yamamoto, N. Ueyama, *Analyst* **2007**, *132*, 358–364.
[28] F. Barragán, P. López-Senín, L. Salassa, S. Betanzos-Lara, A. Habtemariam, V. Moreno, P. J. Sadler, V. Marchán, *J. Am. Chem. Soc.* **2011**, *133*, 14098–14108.
[29] K. Adamson, C. Dolan, N. Moran, R. J. Forster, T. E. Keyes, *Bioconjugate Chem.* **2014**, *25*, 928–944.
[30] L. He, Y. Huang, H. Zhu, G. Pang, W. Zheng, Y.-S. Wong, T. Chen, *Adv. Funct. Mater.* **2014**, *24*, 2754–2763.
[31] S. L. Goodman, H. J. Grote, C. Wilm, *Biology Open* **2012**, *1*, 329–340.
[32] J. Husson, J. Dehaudt, L. Guyard, *Nat. Protocols* **2014**, *9*, 21–26.
[33] S. Bonnet, J.-P. Collin, N. Gruber, J.-P. Sauvage, E. R. Schofield, *Dalton Trans.* **2003**, 4654–4662.
[34] T. G. Kapp, M. Fottner, O. V. Maltsev, H. Kessler, *Angew. Chem. Int. Ed.* **2016**, *55*, 1540–1543; *Angew. Chem.* **2016**, *128*, 1564–1568.
[35] a) O. Novakova, J. Kasparkova, O. Vrana, P. M. van Vliet, J. Reedijk, V. Brabec, *Biochemistry* **1995**, *34*, 12369–12378; b) D. Lazic, A. Arsenijevic, R. Puchta, Z. D. Bugarcic, A. Rilak, *Dalton Trans.* **2016**, *45*, 4633–4646.

Received: September 4, 2016
Published Online: December 2, 2016

