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# Functionalization of Ruthenium(II) terpyridine complexes with cyclic RGD peptides to target integrin receptors in cancer cells

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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) via amide bond formation resulting in a monomeric and a dimeric bioconjugate. Both Ru(II) complexes bind strongly and selectively to integrin  $\alpha_{v}\beta_{3}$ , with the dimeric molecule displaying 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.<sup>[1]</sup> 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,<sup>[2]</sup> gold,<sup>[3]</sup> iron<sup>[4]</sup> and copper<sup>[5]</sup>. Thus, the development of so-called targeting and drug delivery strategies of metallodrugs has

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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.<sup>[6]</sup> Specifically, the functionalization of metallodrugs is aimed at improving the tumor selectivity and/or minimizing the systemic toxicity to enhance their cellular accumulation and 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 allow to visualize the drug molecules *in vitro* or *in vivo*, thus leading to the design of theranostic agents.<sup>[7]</sup>

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

Integrin receptors have been largely explored as drug targets since they are heterodimeric, transmembrane receptors that function as mechanosensors, adhesion molecules and signal transduction platforms in a multitude of biological processes.<sup>[9]</sup> Integrins interact with the extracellular matrix (ECM) thereby regulating many cellular functions, such as proliferation, migration, and survival. Integrins are also involved in the cell-tocell interactions. Through cell-cell and cell-ECM contacts, the integrins transduce the information from the external environment into the cell and vice-versa, to promote cell adhesion, spreading and motility.<sup>[10]</sup> One common feature of the integrin family is a heterodimeric structure that consists of  $\alpha$  and  $\beta$  subunits.<sup>[11]</sup> 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,<sup>[12]</sup> being upregulated in tumor cells compared to low levels in normal endothelial cells. The integrin receptor  $\alpha_{v}\beta_{3}$  plays a crucial role in these processes<sup>[13],[14]</sup> and became 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.<sup>[16]</sup> In fact, eight of the above mentioned integrin subtypes form the RGD-binding class.<sup>[17]</sup> Since then, a wide screening of peptide libraries has been carried out to discover ligands including the RGD sequence, and targeting the integrin receptors with even higher selectivity. Interestingly, the cyclic pentapeptide cyc[RGDfK] (Figure 1) was found to have increased selectivity for integrin  $\alpha_v \beta_3$ .<sup>[18]</sup>

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**Figure 1.** Cyclic pentapeptide cyc[RGDfK] (**A**) and two representative targeted Pt constructs: a Pt(IV) conjugate<sup>[19]</sup> (**B**) and nanoparticles encapsulating a cisplatin prodrug<sup>[20]</sup> (**C**).

Among the metal-based radiopharmaceuticals tethered to cyclic RGD peptides, the majority of the examples reported in the literature have been evaluated as SPECT and PET radiotracers for tumor imaging.<sup>[7a, 21]</sup> Recently, the preclinical evaluation of the potential theranostic radiopharmaceutical <sup>66</sup>Ga-DOTA-E(cyc[RGDfK])<sub>2</sub> compound has been reported.<sup>[22]</sup>

As an example of targeted anticancer metal complexes, recent reports describe the synthesis and biological evaluation of Pt(IV) prodrugs, whose axial positions could be functionalized with cyclic RGD tripeptides that bind selectively to the integrin receptor  $\alpha_v\beta_3$ .<sup>[[19, 23]</sup> 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.<sup>[20]</sup>

Concerning anticancer ruthenium complexes coupled to peptides, some examples have been already reported in the literature,<sup>[8]</sup> including luminescent Ru(II) complexes linked through the mitochondrial penetrating peptide (MPP),<sup>[24]</sup> as well as to the nuclear localization sequence (NLS),<sup>[25]</sup> the latter enabling 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 imaging of key cell organelles.<sup>[26]</sup> Ueyama et al. also described a peptide labeling approach using Ru(II) terpyridine complexes, to implement the mass spectrometry detection of proteolytic peptides.<sup>[27]</sup>

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<sup>[28]</sup>, which dissociates from the peptide by irradiation with visible light to form an aqua complex that generates monofunctional adducts with guanine bases of the DNA. Furthermore, Adamson *et al.* designed luminescent Ru(II) polypyridyl complexes attached to the linear RGD tripeptide, acting as molecular probes for reporting on the presence and conformation of integrins.<sup>[29]</sup> 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 have been attached to RGD-functionalized mesoporous silica nanoparticles,<sup>[30]</sup> whose uptake and sub-cellular distribution could be followed by fluorescence microscopy. Interestingly, the RDG peptide on the nanoparticle surface induces an increased selectivity for cancer cells. After internalization of the nanoparticle, the ruthenium species are released and induce changes in the phosphorylation pathway of certain protein kinases leading to apoptosis.

To the best of our knowledge, besides the above mentioned publications, there are no further studies about 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 via amide bond formation. Thus, the compounds were tethered to one or two peptides. In the latter case, anchoring to two cyc[RGDfK] were intended to enhance the binding affinity to the  $\alpha_{v}\beta_{3}$  integrin receptor.<sup>[13]</sup> The binding affinities of the ruthenium-RGD conjugates for both the  $\alpha_{v}\beta_{3}$  and  $\alpha_{5}\beta_{1}$  integrin receptors have been 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  $\alpha_{\nu}\beta_{3}$  integrin expression) and human mammary carcinoma SKOV3 cells (moderate  $\alpha_v \beta_3$  integrin expression).<sup>[31]</sup>

#### **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 has been followed.<sup>[32]</sup> 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<sub>4</sub> to obtain [2,2':6',2"-terpyridine]-4'-carboxylic acid (terpy\*, **1b**) (see Scheme 1).

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Scheme 1. Synthesis of ligand 1b, [2,2':6',2"-terpyridine]-4'-carboxylic acid.



The complexes **3a** and **3b** were prepared by a novel synthetic route based on literature procedures<sup>[33]</sup> (Scheme 2). Heating RuCl<sub>3</sub>·3H<sub>2</sub>O with **1a** or **1b** in dry ethanol yields the brown complexes **2a** and **2b**, respectively, after one hour in the dark. Afterwards, the complexes reacted with **1b**, triethylamine and LiCl for chloride abstraction and reduction of Ru(III) to Ru(II). Upon addition of 1 M KPF<sub>6</sub> solution, the complexes [Ru(terpy)(terpy\*)](PF<sub>6</sub>)<sub>2</sub> (**3a**) and [Ru(terpy\*)<sub>2</sub>](PF<sub>6</sub>)<sub>2</sub> (**3b**) bearing one or two carboxylic acid groups, respectively, precipitate.



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

The conjugation of 3a and 3b to the cyclic peptide cyc[R(Pbf)GD(tBu)fK] was accomplished by reaction of the free carboxylic acid groups of the complexes with the primary amine of the lysine side chain in the presence of a mixture of the activating agents HATU and HOAt (Scheme 3). The success of the bioconjugation reaction was confirmed by Electrospray Ionisation Mass Spectrometry (ESI-MS), which allowed to identify the intermediate products at m/z = 752.78for [Ru(terpy)(terpy-cyc(R(Pbf)GD(tBu)fK))]<sup>2+</sup> and 1221.58 for [Ru(terpy-cyc(R(Pbf)GD(*t*Bu)fK))<sub>2</sub>]<sup>2+</sup>, respectively. Afterwards, the remaining protection groups of Arg and Asp were cleaved 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 since the compounds decomposed during reverse phase (RP)-HPLC. Finally, the products were precipitated by addition of solid KPF<sub>6</sub> to give [Ru(terpy)(terpy-cyc(RGDfK)](PF<sub>6</sub>)<sub>2</sub> (4a) and [Ru(terpy $cyc(RGDfK))_2](PF_6)_2$  (4b) as red solids.



Scheme 3. Synthesis of the bioconjugate products 4a and 4b (PG = protecting group).

Characterization of the ligand 1b complexes 3a,b and 4a,b



Figure 2. <sup>1</sup>H NMR spectra of 1b and complexes 3a and 3b (in DMSO-d6).

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

Comparing the <sup>1</sup>H NMR spectra of ligand 1b with 3a, several signal shifts are observed due to complex formation (Figure 2). The signals of  $H^{3,5'}$  and  $H^{3,3''}$  are shifted downfield around  $\Delta \delta$  = +0.61 or +0.49 ppm. In contrast, the signal of H<sup>4,4"</sup> 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 shift of  $H^{3,5'}$  and  $H^{3,3''}$  is 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 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 and second, the increase of 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 <sup>31</sup>P NMR spectra the presence of the PF<sub>6</sub><sup>-</sup> counter ions in complexes 3a and 3b is confirmed by the characteristic septet.

The complexes **3a** and **3b** and their conjugation derivatives **4a** and **4b** were characterized by ESI-MS, where the characteristic isotopic patterns are consistent with the assigned structures (Figure S3-S14 in the supplementary material). The ESI-MS spectra of the complexes show signals at 757.05 and 306.04 m/z for **3a** and 801.04 and 328.04 m/z for **3b**, which indicate the loss of one or two PF<sub>6</sub><sup>-</sup> anions, leading to a single or double positive charge cationic species. Similarly, for the coupling products **4a** and **4b**, the loss of PF<sub>6</sub><sup>-</sup> anions is observed. The characteristic isotopic patterns of the signals match perfectly with the calculated ones, which can be seen in the supporting information.

Integrin binding assay

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The impact of the conjugation of 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<sup>[34]</sup> are shown in Table 1.

**4a** exhibits an IC<sub>50</sub> value of 49 ± 4.3 nM, 90 fold higher than Cilengitide (0.54 ±0.06 nM). However, the selectivity for  $\alpha_v\beta_3$  is reasonably high reflecting the fact that the bioconjugate does not bind the  $\alpha_5\beta_1$  receptor at all (IC<sub>50</sub> > 1000 nM), while Cilengitide has still an affinity of 15.4 ± 0.2 nM. Considering bioconjugate **4b**, enhanced binding affinities are predicted due to its dimeric character. Indeed, the binding affinity for integrin  $\alpha_v\beta_3$  is 2.5 ± 0.3 nM, presenting a 20 times higher affinity than that observed for the monomeric product and nearly approaching the value of Cilengitide. Since the affinity for the  $\alpha_5\beta_1$  receptor shows merely a value about 595 ± 67 nM, the high selectivity of **4b** for  $\alpha_v\beta_3$  is demonstrated.

Table 1. Results of integrin binding assays for the bioconjugates 4a and 4b, in comparison to the benchmark Cilengitide.  $^{[a]}$ 

|                             | IC <sub>50</sub> [nM] ± SD |                   |
|-----------------------------|----------------------------|-------------------|
| Compound                    | $\alpha_{\nu}\beta_{3}$    | $\alpha_5\beta_1$ |
| Cilengitide <sup>[34]</sup> | 0.54 ± 0.06                | 15.4 ± 0.2        |
| 4a                          | 49 ± 4.3                   | >1000             |
| 4b                          | $2.5 \pm 0.3$              | 595 ± 67          |

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

#### Antiproliferative activity

The ruthenium compounds (**3a** and **3b**) and their 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 integrins  $\alpha_v\beta_3$ .<sup>[31]</sup> Unfortunately, both the ruthenium(II) complexes and their targeted derivatives show 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 are observed.

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

|          | IC <sub>50</sub> [µM] <sup>[a]</sup> |             |
|----------|--------------------------------------|-------------|
| Compound | 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  $\pm$  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 have been prepared in order to deliver anticancer metallodrugs directly to tumors cells overexpressing the  $\alpha_v\beta_3$  integrin receptor.

The preparation of the terpy-based ruthenium complexes 3a and 3b bearing one or two carboxylic acid groups, respectively, was carried out using a novel synthetic strategy. The compounds were coupled to a protected derivative of the cyclic pentapeptide via amide bond formation between the carboxylic acid of the complex and the amine group of the lysine side chain. Purification of the resulting monomeric (4a) or dimeric (4b) bioconjugates and was achieved by Size Exclusion Chromatography followed by precipitation as PF<sub>6</sub>-salt. 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 due to still scarce uptake in cancer cells. Hence, while the reported strategy holds promise to achieve targeted metallodrugs, future studies have to focus on the 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].

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**Keywords:** tumor targeting peptide • ruthenium • antitumor agent • bioconjugation • RGD

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Two terpyridine-based ruthenium(II) complexes were designed to be coupled to a cyclic peptide targeting integrin receptor  $\alpha_v\beta_3$  in order to achieve models for targeted anticancer agents.



#### Targeting drugs

Eva M. Hahn, Natalia Estrada, Jiaying Han, Vera F. C. Ferreira, Tobias G. Kapp, João D. G. Correia, Angela Casini<sup>\*</sup> and Fritz E. Kühn<sup>\*</sup>

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Functionalization of Ruthenium(II) terpyridine complexes with cyclic RGD peptides to target integrin receptors in cancer cells