Non-conventional \textit{trans}\---Platinum Complexes Functionalized with RDG

Peptides: Chemical and Cytotoxicity Studies

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Abstract: In order to target platinum complexes to cancer cells, \textit{trans}-Pt(II) or \textit{trans}-Pt(IV) complexes were bioconjugated to the cyclic peptide cRGDK (cRGD), with affinity for a $\alpha\beta_3$ integrin receptors, through their 4-picolinic acid spectator ligands. To tackle this goal, the Pt(II) and Pt(IV) precursors were activated at their carboxylic acid function and further reacted with the cRGDK peptide, to afford the bioconjugates Pt(II)-cRGD and Pt(IV)-cRGD, respectively. Pt(II)-cRGD was studied by \textsuperscript{195}Pt-NMR that confirmed the presence of the Pt(II) center. In contrast, the characterization of Pt(IV)-cRGD was not possible due to the tendency of this complex to undergo reduction to Pt(II) in solution. Thus, only the Pt(II)-cRGD complex was used for further biological studies, and it exhibited some cytotoxic activity against the HUVEC cell line, with the highest levels of a $\beta_3$ expression. However, no improved effects were observed with respect to the Pt(II)-pic precursor. Studies by ICP-MS showed enhanced intracellular accumulation for Pt(II)-cRGD with respect to Pt(II)-pic in cancer cells. Overall, these results show that while Pt(II) bioconjugation enhances compound’s uptake, it did not translate into an increase in cytotoxicity.

Introduction

Metal-based anticancer agents are major components in the chemotherapeutic regime of many tumor diseases. Platinum complexes are among the most used metallodrugs due to the widespread usage of cisplatin in the clinical setting. However, the clinical efficiency of cisplatin is hampered due to its low solubility, side effects, and poor activity versus certain tumors (tumor resistance).\textsuperscript{[1]} In view of these limitations, research has been extended to new platinum analogues with the aim of overcoming these unwanted effects.\textsuperscript{[2]} Most of this research has been performed with cis configuration because initial research reported a lack of activity of transplatin. However, several exceptions for non-conventional \textit{trans}-[PtCl\textsubscript{2} (L\textsubscript{2})] complexes were reported, indicating that synthetic variations in the coordinating ligands could lead to new active species.\textsuperscript{[3]} Based on aliphatic amines as spectator ligands, our research was also able to demonstrate that active \textit{trans} Pt(II) complexes were achievable.\textsuperscript{[4, 5]}

Both for cisplatin and trans-platinum like complexes, drug targeting strategies are considered important tools to enhance their selectivity and efficacy as antitumoral agents. Receptors associated with cell growth and division are often overexpressed in tumors, being therefore relevant for drug targeting. In particular, peptide receptors are overexpressed in certain tumors, as compared to endogenous expression levels, being such receptors relevant targets to achieve a specific accumulation of metallodrugs in tumor cells.\textsuperscript{[6]} The examples of Pt compounds carrying bioactive peptides for specific delivery of Pt remain scarce. Reedijk et al have proved how platinum complexes with a broad library of dipeptides and tripeptides showed cytotoxic activity against cancer cell lines. Although such activity was not higher than cisplatin, structural variations on this tested series demonstrated a clear influence in the activity of the series.\textsuperscript{[7-9]}

With the same aims, Metzler-Nolte and coworkers achieved different series of metal compounds’ bioconjugates using cell penetrating peptides and other examples in a drug targeting and delivery strategy.\textsuperscript{[10, 11]}

Other examples found in the literature have explored a different series of receptors: the integrin family. Integrins are heterodimeric receptors that play a pivotal role in many cell--cell and cell--extracellular matrix interactions.\textsuperscript{[12, 13]} They consist on transmembrane glycoproteins that contain two non-covalently bound $\alpha$ and $\beta$ subunits. The integrin receptor $\alpha\beta_3$, also known as the vitronectin receptor, is expressed in endothelial cells and modulates cell migration and survival during angiogenesis. Being overexpressed in a variety of tumor cell types, such as glioblastoma, melanoma, ovarian, breast and prostate cancer, it potentiates tumor invasion and metastasis.\textsuperscript{[14]} The $\alpha\beta_3$-integrin recognizes selectively extracellular matrix proteins, such as vitronectin or fibronectin, which contain the exposed Arg-Gly-Asp (RGD) sequence.\textsuperscript{[15]} The discovery of the canonical RGD sequence motivated an intense research work on small peptide-based molecules aimed at targeted drugs for antiangiogenic therapy.

Lippard et al used an approach exploring the targeting ability of peptides such as linear and cyclic RGD and Asn-Gly-Asp (NGR), and synthesized a Pt(IV) series comprising mono and bifunctionalized complexes, which positively improved the cisplatin activity as a consequence of the peptide release once the Pt(IV) compound reduces to cisplatin (Pt(II)).\textsuperscript{[16]} More recently, other Pt(IV) complexes functionalized with cyclic and multimeric RGD containing-peptides have been described. Interestingly, conjugation of a photoactivatable Pt(IV) pro-drug to a cyclic RGD-containing peptide, led to increased phototoxicity in melanoma cancer cells overexpressing the $\alpha\beta_3$-integrin receptor.\textsuperscript{[17]} Other approach explored the possibility of enhancing the selectivity and potency of a Pt(IV) derivative of...
picoplatin by the conjugation with monomeric and multimeric cRGD peptides.\(^{[18]}\)

Previously, we have shown that some non-classical trans-Pt(II) complexes with isopropylamine (ipa) and pyridine derivatives are active in cisplatin-resistant cell lines,\(^{[4, 5]}\) as well as the corresponding Pt(IV) counterparts. Herein, we describe our attempts to functionalize these Pt(II)/Pt(IV) complexes with the cyclic RGD peptide cRGDfK, aiming to obtain compounds with enhanced selectivity towards their cytotoxic action (Figure 1).

The same strategy was used to functionalize the Pt(II) and Pt(IV) precursors with the RGD derivative, which comprised the prior activation of the carboxylic function of the 4-picolinic acid coordinated to Pt(II) or Pt(IV), followed by the subsequent coupling of the peptide. Hence, the synthesis of the metallated bioconjugates started with the peptide synthesis by a two-step method. First, the automated synthesis of the linear RGD peptide was achieved in solid-phase, followed by the cyclization in solution using strategies reported before.\(^{[21]}\) The final cyclic peptide was characterized by mass spectrometry.

We have taken into consideration that cyclic RGD peptides are more efficient in targeting endothelial cells and have been extensively used for research, therapy, and diagnosis of neoangiogenesis.\(^{[19]}\) Additionally, cRGDfK in particular, can be easily attached to the metal moieties via the lysine side chain, while preserving its biological activity.\(^{[19, 20]}\)

To achieve our goal, we hypothesized that the conjugation of cRGDfK to the trans-Pt(II) or trans-Pt(IV) complexes, through a 4-picolinic acid spectator ligand, would lead to Pt bioconjugates with the ability of specifically recognizing cells overexpressing \(\alpha_v\beta_3\) receptors, with a consequent enhancement of the cytotoxic activity against the same cell lines.

**Results and Discussion**

**Synthesis and Characterization**

By studying the functionalization of related Pt(II) and Pt(IV) complexes with the same cRGDfK derivative, we have considered that the intracellular reduction of the RGD-containing Pt(IV) pro-drug could contribute to further improve the selectivity of the cytotoxic action of the Pt(II) unit carrying the bioactive peptide. To clarify this issue, the comparison of the biological behaviour of the resulting Pt(II) and Pt(IV) bioconjugates would be of the greatest importance.

Thereafter, the resulting Pt(II) and Pt(IV) activated esters were reacted with the cRGD derivative to afford the final Pt(II) and Pt(IV) bioconjugates. In the case of the Pt(II) bioconjugate, further purification (including HPLC) yielded the desired complex, Pt(II)-cRGD (Scheme 1), as detailed in the experimental section. The chemical identity of Pt(II)-cRGD was confirmed by \(^{195}\)Pt-NMR and mass spectrometry (see Figure 2 and Figure S1).

According to ESI-MS analysis of an aliquot of the reaction mixture, the anchoring of the cRGD peptide to the Pt(IV) complex resulted in the formation of a Pt(IV) bioconjugate, Pt(IV)-cRGD, with the expected molecular ion peak (ESI–MS (+) (m/z): 1144.28 [M+CH\(_3\)CN+H]\(^+\), calc. for C\(_{36}\)H\(_{53}\)Cl\(_4\)N\(_{11}\)O\(_8\)Pt 1102.25 [M]\(^+\); see experimental peaks in Figure S2)

Moreover, the HPLC analysis of the crude reaction mixture showed the presence of a major peak at \(t_R = 25.0\) min, which is clearly different from the retention time exhibited by Pt(II)-cRGD (\(t_R = 19.8\) min) under the same analytical conditions.
Unfortunately, all the attempts to record the $^{195}$Pt-NMR of Pt(IV)-cRGD were not successful (see Figure S3). Nuclear Magnetic Resonance (NMR) long accumulation experiments (overnight) finally afforded a small signal (that became stronger along time) in the Pt(II) area corresponding to the Pt(II)-cRGD complex (see Figures S4 and S5). These findings indicated that Pt(IV)-cRGD is not stable in solution being reduced to its Pt(II)-pic counterpart. For this reason, no further studies were performed with this Pt(IV)-cRGD complex.

Antitumoral activity in human cancer cell lines

The antiproliferative properties of Pt(II)-cRGD and Pt(II)-pic were assayed by monitoring their ability to inhibit cell growth. Cytotoxic activity was determined on different human cancer cell lines: breast carcinoma cells (MDAMB231 and MCF7), ovarian carcinoma cells (A2780 and SKOV3) and human adenocarcinoma (A549). This diverse panel was chosen in order to perform the cytotoxicity evaluation in cell lines of different tumoral origins and with different levels of $\alpha_\beta_3$-integrin expression. Additionally, the non-tumoral HUVEC human endothelial primary cell line overexpressing the $\alpha_\beta_3$-integrin receptor was also tested in the same assay. A comparison between the activity of reference drug cisplatin (CDDP) and the activity of these metal compounds was performed. Using an appropriate range of concentrations, dose-response curves after long-term (72 h) exposure were obtained. From the experimental values, the IC$_{50}$ values for the compounds were calculated (Table 1).

In the human cell lines with no detectable expression of $\alpha_\beta_3$-integrin, the new platinum(II) complex coupled to the cRGD peptide, Pt(II)-cRGD, did not show any measurable cytotoxic activity, neither an increase of such activity compared with the parental complex. In fact, in almost all cell lines tested, the conjugation of the Pt(II) complex to the carrier peptide resulted in a marked reduction of the cytotoxic activity.

Worth of note is that in the HUVEC cell line, which has the highest levels of $\alpha_\beta_3$ expression, the Pt(II)-cRGD complex exhibited some cytotoxic activity. Nevertheless, no marked increase in antiproliferative effects of Pt(II)-cRGD was detected in comparison with the respective parental complex in the HUVEC cells. In fact, the IC$_{50}$ measured for Pt(II)-cRGD is only ~1.4 times higher than the value obtained for Pt(II)-pic under the same conditions. We have reasoned that these overall results could possibly reflect the decreased uptake of Pt(II)-cRGD compared with the parental Pt(II) complex in the cell lines presenting a lower expression of $\alpha_3\beta_3$-integrin receptors, at variance with what has been observed in the case of HUVEC cells, where the compound could undergo an increased uptake due to the overexpression of the same receptors. To clarify this issue, we have performed cell uptake studies for the parental complex (Pt(II)-pic) and respective RGD derivative (Pt(II)-cRGD) in selected cell lines, as described below.

### Table 1. IC$_{50}$ values (expressed in $\mu$M) for Pt(II)-cRGD complex, its precursor and CDDP, as determined by the MTT assay after 72 h of incubation of the compounds at 37 °C with tumoral and non-tumoral cells

<table>
<thead>
<tr>
<th>Cell lines (αβ3 expression)</th>
<th>A2780 (−)</th>
<th>A549 (−)</th>
<th>MCF7 (−)</th>
<th>MDA MB231 (+)</th>
<th>SKOV3 (+)</th>
<th>HUVEC (+++)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt(II)-pic</td>
<td>87.67 ±1.2</td>
<td>&gt;100</td>
<td>60.3 ±1.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>59 ±11</td>
</tr>
<tr>
<td>Pt(II)-cRGD</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>85 ±15</td>
</tr>
<tr>
<td>cCDDP</td>
<td>1.5 ±0.2</td>
<td>10.8 ±0.8</td>
<td>38.2 ±1.3</td>
<td>8.5 ±1.1</td>
<td>13.8 ±1.6</td>
<td>-</td>
</tr>
</tbody>
</table>

### Cellular uptake evaluation

An important parameter in the cytotoxic activity of metallo-drugs is the uptake and accumulation of the metal in cells. In order to rationalize the results of the cytotoxicity studies, and taking into consideration that the new platinum complex is a targeted metal-peptide conjugate, cellular uptake experiments were performed in the MCF7 and HUVEC cells lines. These cells were chosen because they present very different levels of expression of $\alpha_\beta_3$ [22] and, importantly, the parental complex presents similar cytotoxicity values against both cell lines.

After incubation of the cell lines with the complexes for 24 h, cells extracts were collected and the intracellular accumulation of Pt was measured by inductively-coupled plasma mass spectrometry (ICP-MS). The normalized results out of a single experiment are presented in Figure 3. There is a higher accumulation of platinum in HUVEC cells after exposure to both Pt(II)-pic and Pt(II)-cRGD when compared with MCF7 cells. In the case of the parental complex there is 100x more uptake in primary HUVEC cells (0.17 nmol Pt per 10$^5$cells) than in the breast cancer cells (0.0015 nmol Pt per 10$^5$cells), albeit presenting similar IC$_{50}$ values in the two cell lines. Platinum accumulation after exposure to the peptide-metal conjugate was more than 36x times higher in HUVEC cells than in MCF7 cells (1.08 versus 0.0299 nmol Pt per 106 cells). This result is in line with the cytotoxicity studies and with the lower level of expression of $\alpha_\beta_3$ integrin in MCF7 cells compared with HUVEC cells.
It is important to notice that Pt(II)-cRGD showed the greatest increase of cell uptake relatively to the parental complex in the MCF7 cell line, having the lowest expression of the integrin receptor. However, the cell uptake values of both compounds are much higher in the integrin-positive HUVEC cell line, hampering a reliable comparison of the results obtained in the two different cell lines.

Conclusions

The functionalization of a Pt(II)-(ipa) core complex with the cyclic peptide cRGDik has been achieved and the characterization completed. The resulting bioconjugate, Pt(II)-cRGD, did not manifest a substantial increase in cytotoxic activity, as we originally expected, including towards the HUVEC cell line, overexpressing integrin receptors. However, Pt(II)-cRGD’s uptake was substantially increased in cancer cells, and in HUVEC was blocked by excess of cRGD. These results indicate a possible selective and specific tumor uptake for the bioconjugate Pt(II)-cRGD. Further studies will be required to elucidate the intracellular fate and stability of this new platinum complex.

Experimental Section

Reagents and Materials

The platinum complexes trans-[PtCl2(4-picolinic acid)(isopropylamine)], Pt(II)-(ipa) and trans-[PtCl4(4-picolinic acid)(isopropylamine)], Pt(IV)-(ipa) were prepared as described elsewhere.[4, 5] The linear peptide was synthesized using the Fmoc (9-fluorenylmethoxycarbonyl) strategy on a CEM 12-Channel Automated Peptide Synthesizer. Fmoc-amino acids were purchased from CEM. The acid labile 2-chlorotritol chloride resin, benzo triazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop) and 1-hydroxy-1H-benzotriazole (HOBt) were purchased from Novabiochem (Merck), whereas N,N-diisopropylethylamine (DIEA), N-(3-Dimethylaminopropyl)-N'-ethy carbodiimide hydrochloride (EDC.HCl) and N-hydroxy succinimide (NHS) were purchase from Sigma-Aldrich (Spain).

Chromatographic systems

HPLC analyses were performed on a Perkin Elmer LC pump 200 coupled to a Shimadzu SPD 10AV UV/Vis and to a Berthold-LB 509 radiometric detector. Analytical control and semi-preparative purifications of the linear and partially protected RGD and peptide conjugates was achieved on Supelco Discovery Bio Wide Pore C18 (250 x 4.6 mm, 5 μm) and Supelco Discovery Bio Wide Pore C18 (250 x 10 mm, 10 μm) with a flow rate of 1.0 mL min⁻¹ or 2.0 mL min⁻¹ (gradient B), respectively.

Applied binary gradients

Gradient A (Mobile phase: A = aqueous 0.1% CF₃COOH and B = MeOH): 0-3 min, 0% mobile phase B; 3-3.1 min, 0 – 25 % mobile phase B; 3.1-19 min, 25 % mobile phase B; 9.1-20 min, 34 – 100 % mobile phase B; 20-25 min, 100 % mobile phase B; 25-25.1 min, 100 – 0 % mobile phase B; 25.1-30 min, 0 % mobile phase B.

Gradient B (Mobile Phase A = H₂O and B= ACN): 0-3 min, 55-100 % B; 35-37 min, 100 % B; 37-38 min, 100-55% B; 38-40 min, 55 % B.

Gradient C (Mobile Phase A = H₂O and B= ACN): 0–3 min, 0 % B; 3–30 min, 0-50 % B; 30 –33 min, 50 % B; 33–31.1 min, 100 % B; 33.1–37 min, 100 % B; 37-38 min, 100–0 % B, 38-40 min, 0 % B.

Peptide Synthesis

The linear sequence was assembled by solid-phase peptide synthesis (SPPS) on 2-chlorotritol chloride resin according to the Fmoc strategy. After peptide cleavage from the resin under mild acidic conditions (1% TFA/CH₂Cl₂), the solution was concentrated to 5% of the initial volume and a white solid was precipitated with cold water in an ice bath. The partially protected peptide was washed consecutively with water (three times), 5 moisture aqueous NaHCO₃ (twice), water (three times), 0.05 M KHSO₄ (twice), and water (six times) and lyophilized. RP-HPLC (220 nm, gradient B): 95% (tR = 10.4 min). ESI–MS (+) (m/z): 1031.1 [M+H]⁺, calc. for C₄₉H₇₅N₉O₁₃S₂, 1030.24.

Cyclization was performed via in situ activation with 2 equiv. of PyBop and with 10 equiv. of DIEA as solid base under high-dilution conditions (5 mM) in CH₂Cl₂/DMF (11:1) for 16 h. The reaction mixture was purified by semipreparative RP-HPLC (220 nm, gradient A). After evaporation of the solvent, the compounds were deprotected with a standard cocktail mixture (95% TFA, 2.5% triisopropylsilane, 2.5% H₂O) and precipitated with ice-cold diethyl ether. The compound was purified by preparative RP-HPLC (method B). After evaporation of the organic solvent, the compound was lyophilized and characterized by ESI–MS.

RP-HPLC (220 nm, gradient C): 95% (tR = 14.0 min). ESI–MS (+) (m/z): 604.6 [M+H]⁺, calc. for C₂₂H₄₁NaO₇, 603.67.

Figure 3. Cell accumulation of platinum in HUVEC and MCF7 cells after exposure to Pt(II)-cRGD and parental Pt(II) complex (50 μΜ, 24 h). The platinum content is normalized to the cell number. The reported results are from one experiment.

In the case of the HUVEC cell line, we believe that the enhanced intracellular accumulation of Pt(II)-cRGD could be due to its interaction with the targeted receptor at the cell surface. To validate this hypothesis HUVEC cells were incubated with Pt(II)-cRGD in the presence of an excess of free cRGD to block the interaction with αβ₃ integrin. We observed a decrease in intracellular accumulation of ~40% (1.08 versus 0.61 nmol Pt per 10⁶ cells for Pt(II)-cRGD alone and with excess of free cRGD). This result points out for the possible contribution of specific mechanisms of uptake mediated by the αβ₃ receptor.
**General procedure to conjugate RGD to platinum Complexes**

The carboxylic acid of trans-[PtCl2(4-picolinic acid)(isopropylamine)] and trans-[PtCl2(4-picolinic acid)(isopropylamine)] (0.01 mmol) was activated with 1.2 equiv. of EDC.HCl and N-hydroxysuccinimide (NHS) in dry CH2Cl2. After reacting at room temperature for 16 h under nitrogen, the solvent was evaporated and the yellow pall solid was washed with water to remove the urea byproduct. The solid was vacuum-dried. The activated ester were dissolved in CH2CN and added dropwise to a DMF solution (500 µL) containing the cyclo-RGD (0.003 g, 0.005 mmol) and 10 equiv. of NaHCO3. After stirring for ~3 h, the solvent was evaporated and the yellow solid was washed with CH2Cl2, dried, dissolved in water and purified by analytical RP-HPLC. After purification, RP-HPLC analysis (method B) was used to assess the purity (> 95%) of the recovered RGD-containing Pt complexes (see Figure S6).

Cyclo-RGD conjugate-PtIII

RP-HPLC (220 nm, gradient C): tR = 19.8 min. ESI – MS (+) (m/z): 1068.7 [M+Cl]– and 1032.8 [M-H]–. calc. for C53H52Cl2N11O12Pt 1033.86.

δ (195Pt, 64.53 MHz, MeOD-Na2PtCl4): –2380 ppm.

Yield: 10 % (487 µg, 0.0005 mmol; εPhe (257 nm) = 200 L mol⁻¹ cm⁻¹)

Cyclo-RGD conjugate-PtIV

RP-HPLC (220 nm, gradient C): 95% (tR = 25.0 min). ESI – MS (+) (m/z): 1146.8 [M+Cl]–, calc. for C53H52Cl2N11O12Pt 1104.76.

Yield: 6 % (336 µg, 0.0003 mmol; εPhe (257 nm) = 200 L mol⁻¹ cm⁻¹)

**Cell culture**

Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in supplemented EGM medium (Lonza). Human ovarian epithelial cancer cell lines (A2780 and SKOV3) were maintained in RPMI 1640 Medium. Human breast carcinoma cells (MCF-7 and MDA MB 231) and human adenocarcinoma (AS45) were grown in DMEM. RPMI and DMEM culture mediums were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotic solution. All culture mediums and supplements, except when indicated, were from Gibco, Invitrogen, UK. Cells were cultured in a humidified atmosphere of 95% air and 5% CO2 at 37 °C (Heraeus, Germany).

**Cytotoxicity**

The potential as antitumoral agents was explored by the evaluation of drugs • cellular uptake • αvβ3 integrin

of this colored (purple) solution was quantified by measuring the absorbance at 570 nm, using a plate spectrophotometer (Power Wave Xs; Bio-Tek). A blank solution was prepared with DMSO alone (200 µL/well). Each test was performed with at least six replicates. These results were expressed as percentage of the surviving cells in relation with the control incubated without compound. The maximum concentration of DMSO used in compounds solutions (0.1 %) was not cytotoxic. IC50 values were determined using the Graph Pad Prism software and expressed in micromolar concentrations.

**ICP-MS**

To explore the uptake of selected complexes and their intracellular distribution, cells were exposed to each complex (50 µM) for 24 h at 37 °C, and then washed with cold PBS, collected by scraping and centrifuged to obtain a cellular pellet. For the blocking experiments, an excess of cRGD (1mM) was used. The Pt content in the cell pellet was measured, after digestion, by ICP-MS on a ICP-MS NexION 300xx Perkin-Elmer instrument, with 187Rhenium used as internal standard. Briefly, samples were digested with ultrapure HNO3 (65 %), H2O2 and HCl, then evaporated and resuspended in ultrapure water to obtain a 2.0% (v/v) nitric acid solution. The relative standard deviation of the measurements (different aliquots from the same mother solution) was between 0.5 and 2.1%.

**Acknowledgements**

This work was supported by COST action CM1105 (Functional metal complexes that bind to biomolecules), Fundação para a Ciência e Tecnologia (Ph.D. fellowships SFRH/BD/480 66/2008 and SFRH/BD/108623/2015 to M. Morais and V. Ferreira, respectively; project UID/Multi/04349/2014 and FCT Investigator grant to F. Mendes). Accion integrada (PRI-AIBPT-2011-0980 and AI-23/12), SAF2012-34424 and CTQ201568779R are also acknowledged for funding. The authors would also like to thank Célia Fernandes for the Mass Spectrometry analyses, which was carried out on a QITMS instrument, acquired with the support of the Programa Nacional de Reequipamento Científico (Contract REDE/1503/REM/2005- ITN) of FCT and is part of RNEM-Rede Nacional de Espectrometria de Massa. A. G. Quiroga thanks SIDI(UAM) for ICP measurements.

**Keywords:** Platinum complexes • peptides RGD • anticancer drugs • cellular uptake • αvβ3 integrin


[22] S. L. Goodman, H. J. Grote, C. Wilm, Biology Open 2012, 1, 329...