Integrin-targeted delivery into cancer cells of a Pt(IV) pro-drug through conjugation to RGD-containing peptides

Anna Massaguer,*[c] Alejandro González-Cantó,[a] Esther Escribano,[b] Silvia Barrabés,[c] Gerard Artigas,[a] Virtudes Moreno,[b] and Vicente Marchán*[a]

[a]*Departament de Química Orgànica and IBUB, Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain.*

[b]*Departament de Química Inorgànica, Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain.*

[c]*Departament de Biologia, Universitat de Girona, Campus Montilivi, E-17071 Girona, Spain.*

E-mail: vmarchan@ub.edu (Vicente Marchán); anna.massaguer@udg.edu (Anna Massaguer).

ABSTRACT

Conjugates of a Pt(IV) derivative of picoplatin with monomeric (Pt-c(RGDfK), **5**) and tetrameric (Pt-RAFT-{c(RGDfK)}$_4$, **6**) RGD-containing peptides were synthesized with the aim of exploiting their selectivity and high affinity for $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins for targeted delivery of this anticancer metallodrug into tumor cells overexpressing these receptors. Solid- and solution-phase approaches in combination with click chemistry were used for the preparation of the conjugates, which were characterized by high resolution ESI MS and NMR. $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins expression was evaluated in a broad panel of human cancer and non-malignant cells. SK-MEL-28 melanoma cells were selected based on the high expression levels of both integrins, while CAPAN-1 pancreatic cancer cells and 1BR3G fibroblasts were selected as negative control. Internalization experiments revealed a good correlation between integrin expression and the cellular uptake of the corresponding fluorescein-labeled peptides, and that the internalization capacity of the tetrameric RGD-containing peptide was considerably higher than that of the monomeric one. Cytotoxic experiments indicated that the antitumor activity of picoplatin in melanoma cells was increased by 2.6-fold when its Pt(IV) derivative was conjugated to c(RGDfK) (IC$_{50}$ = 12.8 $\pm$ 2.1 µM) and by 20-fold when conjugated to RAFT-{c(RGDfK)}$_4$ (IC$_{50}$ = 1.7 $\pm$ 0.6 µM). In contrast, the cytotoxicity of the conjugates was inhibited in control cells lacking $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin expression. Finally, cellular uptake studies by ICP-MS confirmed a good correlation between the level of expression of integrins, intracellular platinum accumulation and antitumor activity. Indeed, accumulation and cytotoxicity were much higher in SK-MEL-28 cells than in CAPAN-1, being particularly higher in the case of the tetrameric conjugate. The overall results highlight that the great ability of RAFT-{c(RGDfK)}$_4$ to bind to and to be internalized by integrins overexpressed in SK-MEL-28 cells results in higher accumulation of the Pt(IV) complex, leading to a high antitumor activity. These studies provide new insights into the potential of targeting $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins with Pt(IV) anticancer pro-drugs conjugated to tumor-targeting devices based on RGD-containing peptides, particularly on how multivalency can improve both the selectivity and potency of such metallodrugs by increasing cellular accumulation in tumor tissues.

INTRODUCTION

A key challenge in the fight against cancer disease is to develop therapeutic agents that target tumor tissues specifically. Although cancer cells share many common characteristics with normal cells, certain receptors are overexpressed on their surface (e.g. folate receptors, somatostatin receptors, epidermal growth factor receptors, integrins, transferring receptors, etc.), thereby offering an opportunity to deliver cytotoxic agents into human tumors by attachment to a carrier agent.[1] This targeted anticancer strategy can lead to drugs with reduced toxic side effects due to increased tumor selectivity, as well as with improved solubility, biodistribution and ability to cross cell membranes, which are also relevant issues from a therapeutic point of view.

Among receptors overexpressed on tumor cells, integrins are particularly attractive pharmacological targets.[2] These heterodimeric transmembrane cell adhesion glycoproteins have a fundamental role in increasing migration, invasion, proliferation and survival of tumor cells. In addition, integrins have been linked to tumor angiogenesis, which is an essential process for tumor growth and metastasis. Among integrins, the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins are particularly interesting since they are frequently overexpressed in tumor endothelial cells as well as on various tumor cells including lung, breast, melanoma, prostate, ovarian carcinoma and brain tumors.[2a,3] The fact that $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins recognize the consensus tripeptide motif RGD (Arg-Gly-Asp) with high affinity offers the possibility of using carriers based on RGD-containing peptides or peptidomimetics to deliver chemotherapeutic drugs or radionuclides into cancer cells or for tumor imaging purposes.[1b,4] Several studies have demonstrated that the efficiency of the RGD motif to target angiogenic endothelial cells is higher when using constrained peptides embedding the RGD sequence within a cyclic structure.[5] For example, the cyclic pentapeptide c(RGDf[N-Me]V) (Cilengitide)[6], an $\alpha_V\beta_3$ and $\alpha_V\beta_5$ antagonist, is currently in phase III clinical trials as an angiogenesis inhibitor for patients with glioblastoma multiforme. These results have prompted researchers to develop RGD derivatives to be used as tumor-targeting devices of cytotoxic drugs, such as doxorubicin,[7] camptothecin,[8] paclitaxel,[9] or metal-based anticancer drugs.[10] Furthermore, the principle of multivalency[11] has been applied to RGD-containing molecules[12] not only to enhance binding affinity of the ligand to its receptor but

also to promote an active integrin-mediated internalization of the bound entity. In this context, the regioselectively addressable functionalized template (RAFT) cyclodecapeptide scaffold[13] decorated with four copies of the conjugable version of Cilengitide, c(RGDfK), provides a good example on how multivalency can increase the potential of these compounds. Indeed, the tetrameric RAFT-RGD binds 10 times more strongly to the $\alpha_V\beta_3$ integrin than the monomeric RGD peptide, being internalized through an active clathrin-dependent mechanism.[14] Besides offering the possibility to oligomerize four RGD monomers, the well-defined architecture of this cyclodecapeptide scaffold provides a possibility for introducing other substituents such as fluorescent dyes, radionuclide chelators or cytotoxic compounds. In fact, tetrameric RGD-containing derivatives have been used for tumor imaging[15] and for targeted drug delivery[16] by the convenient functionalization of the labeling domain.

Although cisplatin (**1**, Fig. 1) and its derivatives are used in about 50% of all chemotherapeutic regimens administered in the clinic, they cause severe toxic side-effects, mainly owing to their lack of selectivity for cancer cells, which limits the dose that can be administered to patients.[17] Intrinsic or acquired resistance can also limit the scope of the application of Pt(II) metallodrugs in cancer chemotherapy.[18] A promising approach to circumvent these problems consists on increasing the selectivity of platinum drugs against cancer cells by tagging them with carrier molecules whose receptors are overexpressed in the membrane of tumor cells. This targeted approach has the capacity of overcoming some of the problems associated with current metallodrugs by increasing drug efficacy and reducing toxic side-effects.[19] In this context, platinum(IV) complexes,[20] which are considered pro-drugs from a medicinal chemistry point of view, are very attractive compounds since *in vivo* reduction by gluthathione, ascorbate or metallothioneins generates an active square planar Pt(II) complexes by releasing of the axial positions.[21] Attachment of carrier molecules at these positions is particularly interesting since they will be lost upon reduction, thereby avoiding any interference with the mechanism of action of the active Pt(II) species (e.g. interaction with DNA). For this purpose, a Pt(IV) derivative of cisplatin has been derivatized in the detachable axial positions with targeting compounds such as estradiol to target estrogen receptors,[22] folic acid to

target folate receptor,[23] or monomeric RGD-containing peptides to target angiogenic tumors through integrin receptors.[10a,24] The use of pro-drugs based on Pt(IV) complexes offers additional advantages over their Pt(II) congeners, including higher stability in blood which might avoid undesirable interactions with proteins or other biomolecules in blood circulation and results in reduced side-effects, as well as the possibility of oral administration.

Based on the potential of multimeric RGD-containing molecules for tumour imaging and for targeted drug delivery, we wanted to investigate the ability of a tetrameric RAFT-RGD peptide[25] to specifically deliver Pt(IV) complexes into cancer cells through $\alpha_V\beta_3$ and $\alpha_V\beta_5$ cell-surface integrins and to compare it with that of its monomeric version. We reasoned that the higher binding affinity of the RAFT-RGD for the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins may result in higher platinum accumulation in cancer cells compared to the same monomeric ligand and, for instance, in higher cytotoxicity. As a platinum complex, we selected *cis*-ammine(2-methylpyridine)dichloridoplatinum(II) (picoplatin, **2** in Fig. 1), a sterically-hindered platinum compound that was designed to overcome cisplatin resistance mediated by enhanced levels of gluthathione.[26] To this end, the 2-methylpyridine ligand was chosen to hinder the axial approach of nucleophiles such as gluthathione to the platinum center. In recent years, the synthesis and biological evaluation of Pt(IV) derivatives of picoplatin has been explored in an attempt to overcome some of the problems encountered by this compound in phase III clinical trials for the treatment of lung cancer.[27] The oxidation of picoplatin (compound **3** in Fig. 1) resulted in a highly reactive compound[28] whereas the introduction of carboxylato ligands of increasing length resulted in Pt(IV) complexes with activity against drug resistant cell lines.[29] Here, we describe the synthesis, characterization and biological evaluation of the first conjugates between a Pt(IV) derivative of picoplatin and monomeric and tetrameric RGD-containing peptides (Pt-c(RGDfK), **5**, and Pt-RAFT-{c(RGDfK)}$_4$, **6**, respectively; see Fig. 1).
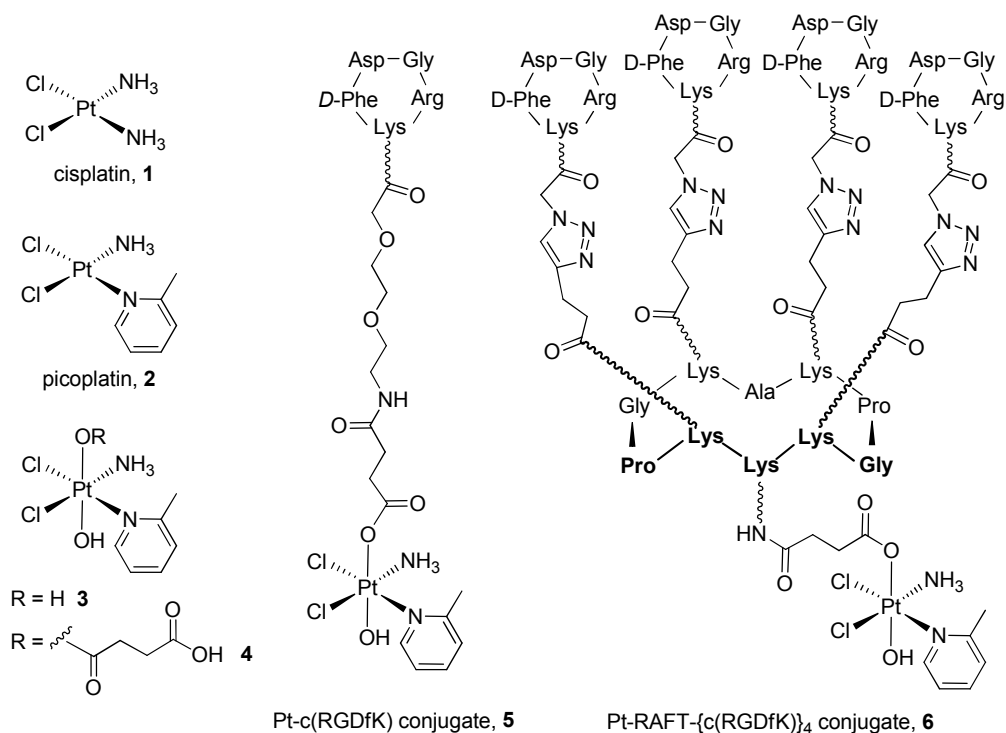
Fig. 1 Structure of cisplatin (**1**), picoplatin (**2**), *cis,cis,trans*-[PtCl$_2$(2-methylpyridine)(NH$_3$)(OH)$_2$] (**3**), *cis,cis,trans*-[PtCl$_2$(2-methylpyridine)(NH$_3$)(OH)(succinate)] (**4**) and of the Pt(IV)-peptide conjugates synthesized (**5** and **6**).

RESULTS AND DISCUSSION

Synthesis and characterization of conjugates between a Pt(IV) derivative of picoplatin and monomeric and tetrameric RGD-containing peptides.

The conjugation of the Pt(IV) derivative of picoplatin was planned via the formation of an amide bond with the peptides, which required the incorporation of a carboxylic function in the Pt complex. In our case, we used *cis,cis,trans*-[PtCl$_2$(2-methylpyridine)(NH$_3$)(OH)(succinate)] (**4**) as the precursor for the synthesis of the conjugates. Since we wanted to compare the effect of monomeric and tetrameric RGD-containing moieties in the biological activity of the resulting conjugates with the picoplatin-based pro-drug, only one axial position was derivatized with a succinate group. Picoplatin was prepared from cisplatin following previously reported procedures.[28,29] The key step involved the preparation of K[PtCl$_3$(NH$_3$)] which was reacted successively in water with KI and 2-methylpyridine to afford *cis*-[PtI$_2$(2-methylpyridine)(NH$_3$)]. This intermediate was easily transformed into picoplatin (**2**).[28] Oxidation of **2** with an excess of hydrogen peroxide (15 mol equiv.) in a 1:10 mixture of water/heptane at 80ºC for 2 h afforded the dihydroxidoplatinum(IV) derivative of picoplatin (**3**) in 51% yield (Scheme 1A). Finally, compound **3** was reacted with succinic anhydride (1.2 mol equiv.) in anhydrous DMF at 40ºC for 2 days. The target complex **4** was obtained as a pale yellow solid after purification by recrystallization (26% yield), and was characterized by ^{1}H NMR and high resolution ESI MS. The homogeneity of the compound was also tested by reversed-phase HPLC analysis.

A solid-phase approach in combination with solution-phase reactions and click chemistry was used for the synthesis of the monomeric (c(RGDfK), **8** in Scheme 1) and tetrameric (RAFT-{c(RGDfK)$_4$}, **11** in Scheme 2)[25] RGD-containing peptides. The linear cyclopentapeptide was assembled on 2-chlorotrityl chloride resin using standard Fmoc/tBu chemistry, cleaved under mild acidic conditions (1% AcOH for 30 min) and cyclized with PyBOP to afford the protected peptide, c[-Arg(Pbf)-Gly-Asp(tBu)-*D*-Phe-Lys(Alloc)-] (**7**). After removal of the Alloc group by treatment with phenylsilane and Pd(PPh$_3$)$_4$,[25] the free ε-NH$_2$ of the lysine residue was used to derivatize the peptide with a polyethyleneglycol spacer. Finally, the Fmoc group and the remaining side chain protecting groups

(tBu and Pbf) were successively eliminated by treatment with 5% piperidine in DMF and TFA/TIS/H$_2$O 95:2.5:2.5, respectively. Peptide **8** was purified by reversed-phase HPLC and characterized by HR ESI-MS.
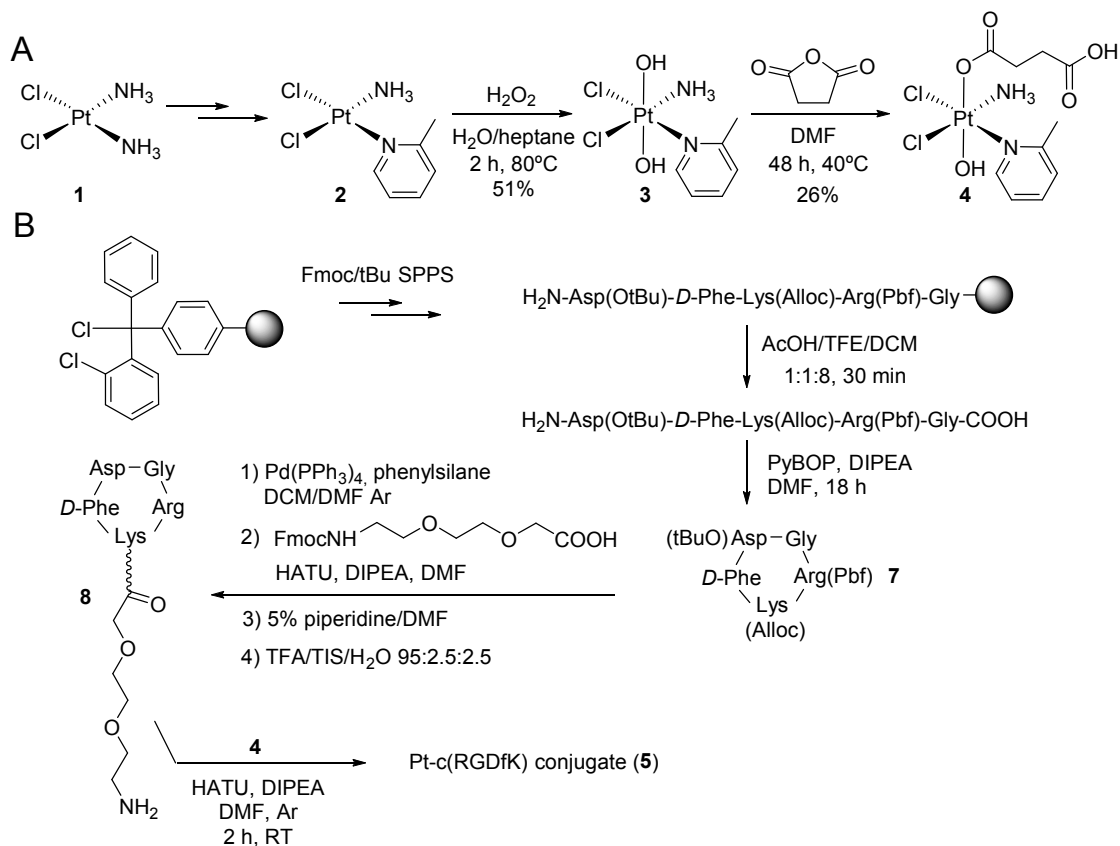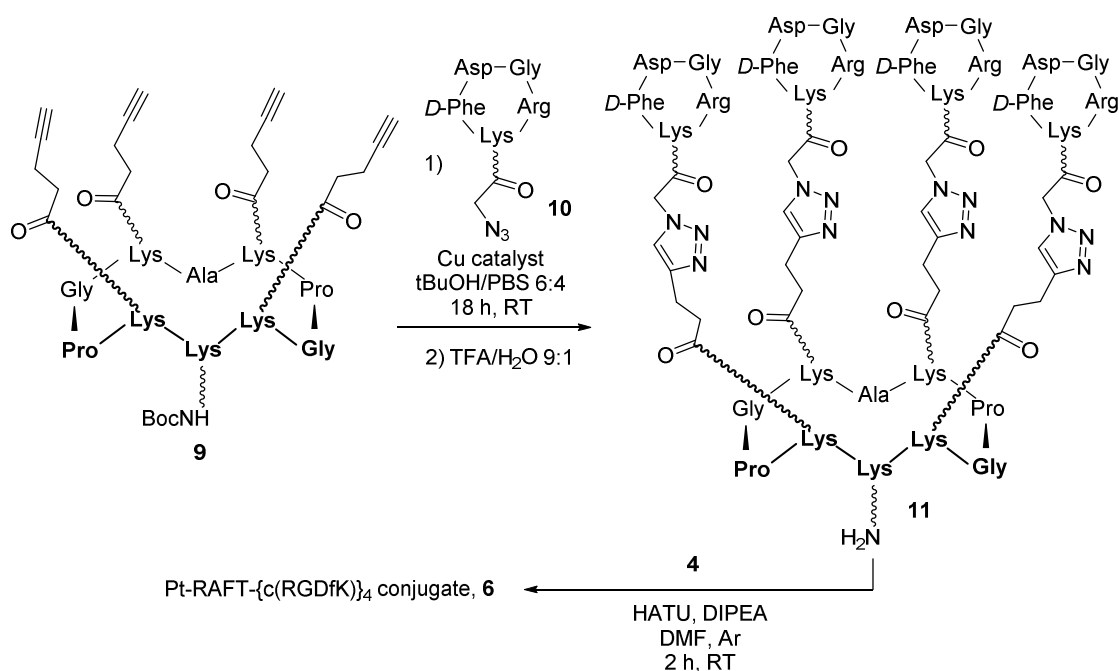

Scheme 1. Schematic representation of the approach used for the synthesis of the Pt(IV) derivative of picoplatin (**4**) (A) and of its conjugation to the monomeric c(RGDfK) peptide (**8**) to afford conjugate **5** (B).

For the synthesis of the tetrameric RAFT-RGD peptide (**11**), an elegant approach recently reported by Galibert et al. was chosen, which makes use of copper(I)-catalyzed azide-alkyne [3+2] cycloaddition for the assembly of the four RGD peptides onto the RAFT cyclopeptide scaffold.[25,30] Following the procedure reported for peptide **7**, the cyclic decapeptide c[-Lys(alkyne)-Lys(Boc)-Lys(alkyne)-Pro-Gly-Lys(alkyne)-Ala-Lys(alkyne)-Pro-Gly-] (**9**) was prepared by using a Fmoc-Lys-OH derivative whose ε-NH$_2$ function had been derivatized with an alkyne group. This RAFT scaffold was reacted with cyclo-pentapeptide **10**, c[-Arg(Pbf)-Gly-Asp(tBu)-D-Phe-Lys(COCH$_2$N$_3$-],[25] bearing the required azide function for click chemistry assembly (Scheme 2). Taking into account previous results reported by Galibert et al.,[25] we used copper nanosize powder as a catalyst for the azido-alkyne cycloaddition during 18 h at room temperature. After elimination of the Boc group and

HPLC purification, the desired RAFT-{c(RGDfK)}$_4$ (**11**) was obtained as a white solid and characterized by HR ESI-MS.


Scheme 2. Schematic representation of the approach used for the synthesis of RAFT-{c(RGDfK)$_4$} (**11**) and its conjugation to the Pt(IV) derivative of picoplatin to afford conjugate **6**.

For the synthesis of the conjugates, the Pt(IV) derivative of picoplatin (**4**) was activated with HATU and DIPEA in anhydrous DMF for 2 min, and allowed to react with the corresponding RGD-containing peptides (**8** or **11**) for 2 h at room temperature. In both cases, analysis by reversed-phase HPLC showed main peaks (see Figures S5 and S7 in the Supporting Information) which were isolated and unambiguously characterized by high-resolution ESI mass spectrometry as the expected products. After purification by semipreparative HPLC and lyophilization, the trifluoroacetate salts of conjugates **5** (overall yield from **8**: 52%) and **6** (overall yield from **11**: 27%) were obtained as white solids.

High-resolution ESI MS analysis of both conjugates afforded m/z values that were consistent with the calculated value of the charged species ([M+H]$^+$ for **5**, and [M+3H]$^{3+}$, [M+4H]$^{4+}$ and [M+5H]$^{5+}$ for **6**) and with the proper isotopic mass distribution patterns of platinum (see Fig. 2 and Fig. S6 and S8 in the Supporting Information). In addition, the monomeric conjugate **5** was characterized by ^{1}H NMR spectroscopy by using 1D and 2D experiments (TOCSY). The aromatic region of the ^{1}H NMR spectra is shown in Fig. 2, where some diagnostic signals from the platinum complex (2-

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methylpyridine) and from the peptide moiety (NH protons of amide bonds and aromatic protons of *D*-phenylalanine residue) are indicated.
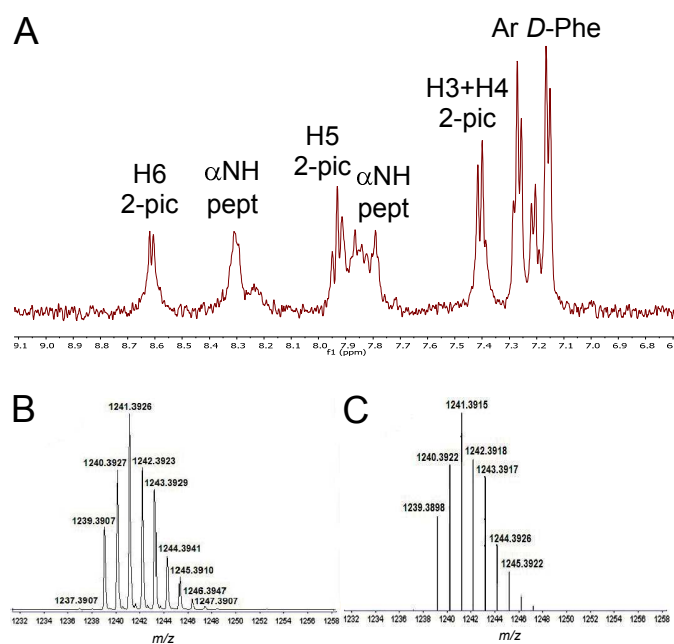

Fig. 2 ^{1}H NMR spectra of conjugate **5** (A) in H_2O/D_2O 9:1, showing the region between 6.7 and 9.1 ppm. Expanded HR ESI mass spectrum of the molecular peak of **5** ($[M+H]^+$), experimental (B) and calculated (C).

Selection of cell lines overexpressing $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins.

To assess the capacity of the monomeric and tetrameric RGD-containing peptides to selectively deliver the Pt(IV) pro-drug into cancer cells, we first focused on studying the level of expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins in a wide panel of cell lines, including cancer cells of different origins and non-malignant cells. The integrin's expression level on the cell surface was characterized by flow cytometry using specific monoclonal antibodies (Fig. 3 and Fig. S14 in the Supporting Information). We found that SK-MEL-28 cells expressed high levels of both $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins (mean cell fluorescence intensity of 156.0 and 13.8, respectively), which is in good agreement with literature reporting that integrin $\alpha_V\beta_3$ is strongly induced on metastatic melanoma cells, while is expressed at low levels on normal melanocytes.[31] In contrast, we detected a very low $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin expression in CAPAN-1 cells (mean cell fluorescence intensity of 4.7 and 5.0, respectively) and in 1BR3G non-malignant cells (mean cell fluorescence intensity of 6.2 and 6.7, respectively). These results indicate that the melanoma cancer cell line is a good model to evaluate the biological activity

of the conjugates. In addition, human fibroblast cells and pancreatic cancer cells can be used as negative control since the expression of both integrins is very low.
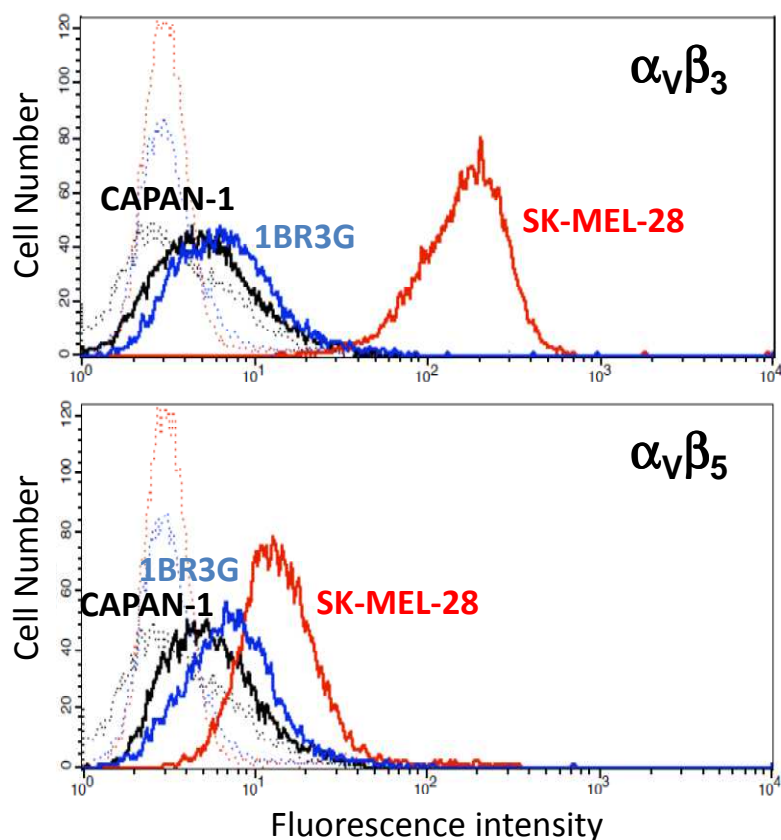

Fig. 3 Expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins on SK-MEL-28, CAPAN-1 and 1BR3G cell lines. Representative flow cytometry histograms obtained after the indirect immunofluorescence staining of the cells. Solid lines represent the fluorescence intensity of the cells after the incubation with monoclonal antibodies against $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins followed by the incubation with secondary antibody conjugated to Alexa-Fluor 488. Dotted lines indicate the background staining with the secondary antibody alone.

Intracellular uptake of fluorescein-labeled RGD-containing peptides (12 and 13).

As previously stated, binding affinity of multimeric RGD-containing peptides toward $\alpha_V\beta_3$ integrin is considerably higher than that of the monomeric versions. For example, a tetrameric RAFT-RGD peptide assembled by oxime ligation and labeled with Cy5 dye binds specifically to $\alpha_V\beta_3$ integrin with a ten-fold higher affinity than c(RGDfK), as measured by fluorescence correlation spectroscopy.[12] In addition, RAFT-RGD is actively and efficiently internalized with $\alpha_V\beta_3$ integrin via clathrin-mediated endocytosis, whereas the monomeric peptide is internalized without modifying

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$\alpha_V\beta_3$ internalization. In order to check the capacity of internalization of c(RGDfK) and of its tetrameric version assembled by click chemistry, RAFT-{c(RGDfK)$_4$},[25] in the selected cell lines, we labeled them with fluorescein. Reaction of the *N*-hydroxysuccinimide ester of 6-[fluorescein-5(6)-carboxamido]hexanoic acid with peptides **8** or **11** in a 1:1 mixture of ACN and aqueous phosphate buffer afforded the expected fluorescein-labelled derivatives in high purity, Fluo-c(RGDfK) (**12**) and Fluo-RAFT-{c(RGDfK)}$_4$ (**13**), respectively, which were purified by reversed-phase HPLC (see Fig. S-2 and S-4 in the Supporting Information) and characterized by HR ESI-MS. Next, flow cytometry was used to obtain quantitative data on the internalization capacity of both peptides. As expected from the higher affinity of the tetrameric RAFT-RGD peptides for integrins, the intracellular uptake of **13** by SK-MEL-28 cells was more elevated compared with that observed in cells incubated with **12**, by 5.1-fold when incubated at 10 μM (mean fluorescence intensity of 74.67 $\pm$ 10.0 *vs* 14.6 $\pm$ 1.8) and by 5.9-fold at 25 μM (128.86 $\pm$ 17.3 *vs* 21.7 $\pm$ 2.08) (Fig. 4A). As represented in Fig. 4B, a similar trend was observed in CAPAN-1 and 1BR3G cells, which showed a 2.2-fold (34.1 $\pm$ 3.2 *vs* 15.6 $\pm$ 0.1) and a 6.0-fold (78.3 $\pm$ 1.8 *vs* 13.0 $\pm$ 1.5) higher uptake efficiency for **13** than for **12** (at 25 μM), respectively. Interestingly, the internalization of peptides **12** and **13** was higher in the integrin overexpressing SK-MEL-28 cells than in CAPAN-1 (by 1.4-fold and by 3.7-fold, respectively) or in 1BR3G cells (by 1.6-fold for both peptides). All together, these results demonstrate that RGD-containing peptides, particularly the RAFT-RGD derivative, display the internalization properties required to deliver the cytotoxic metallodrugs into $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin overexpressing cancer cells in a selective manner.
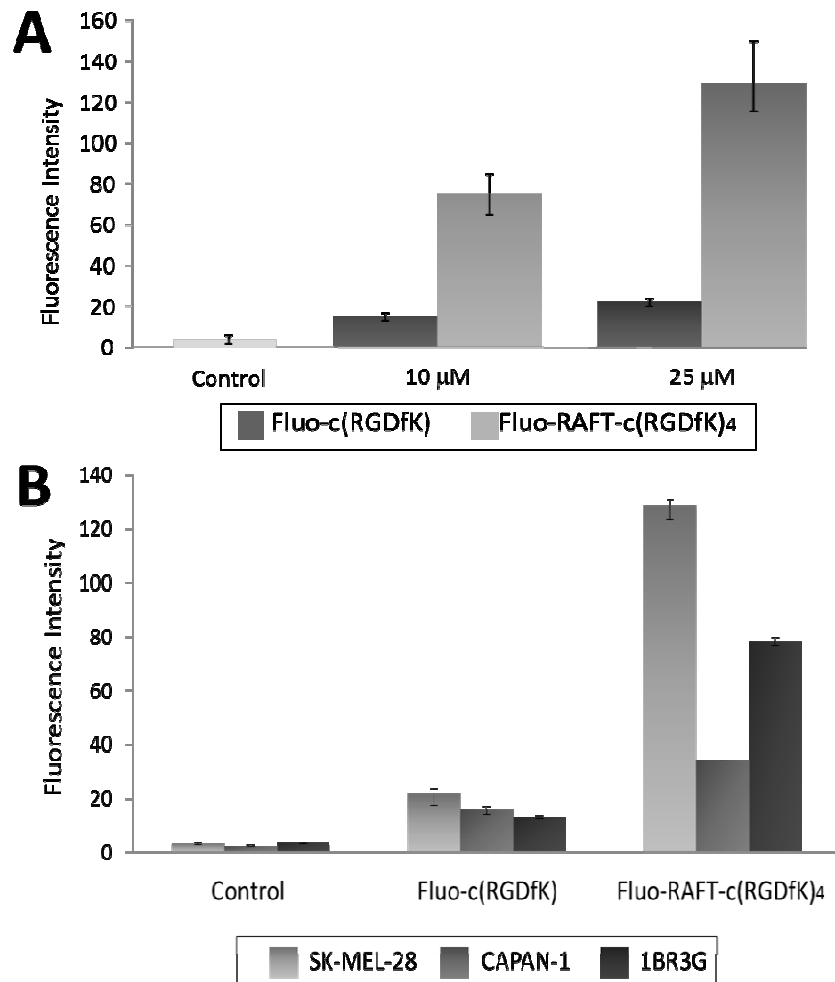
Fig. 4 Intracellular delivery efficiencies of fluorescein-labelled RGD-containing peptides, Fluo-c(RGDfK) (**12**) and Fluo-RAFT-{c(RGDfK)}$_4$ (**13**), in SK-MEL-28, CAPAN-1 and 1BR3G cells. Cells were incubated with the peptides for 1 h or medium alone (control). The fluorescence intensity of the cells, corresponding to the intracellular uptake of the peptides, was determined by flow cytometry. A: Comparison of the intracellular uptake of **12** and **13** in SK-MEL-28 at two different concentrations (10 or 25 µM). B: Comparison of the intracellular uptake of **12** and **13** at 25 µM in SK-MEL-28, CAPAN-1 and 1BR3G cells. Each column in the graphs represents the mean fluorescence intensity of three independent experiments ± SD.

Cytotoxicity studies.

Our next objective was to evaluate the cytotoxicity of the Pt(IV)-peptide conjugates, Pt-c(RGDfK) (**5**) and Pt-RAFT-{c(RGDfK)}$_4$ (**6**), to determine their potential as anticancer agents. Their anti-proliferative activity was determined in SK-MEL-28, CAPAN-1 and 1BR3G cell lines by using the MTT assay that measures mitochondrial dehydrogenase activity as an indication of cell viability. The cytotoxicities of cisplatin, picoplatin and the unconjugated peptides (**8** and **11**) were also determined as a control. As indicated in Table 1, the cytotoxic activity of picoplatin in SK-MEL-28 cells was enhanced by conjugation to both peptides. Conjugate **5** displayed an IC$_{50}$ value of 12.8 $\pm$ 2.1 μM, what represents a 2.6-fold increase of the picoplatin cytotoxicity in this cell line (IC$_{50}$ = 33.6 $\pm$ 6.6). This effect was considerably higher for conjugate **6** (IC$_{50}$ = 1.7 $\pm$ 0.7) since the cytotoxicity against SK-MEL-28 cells was increased by approximately 20-fold when compared to the unconjugated picoplatin. Despite the fact that picoplatin cytotoxicity in SK-MEL-28 cells is lower than that of cisplatin (about 2-fold), conjugation of the Pt(IV) derivative of picoplatin to the tetrameric RAFT-RGD peptide leads to a compound with a much higher cytotoxic activity than cisplatin (IC$_{50}$ = 17.0 $\pm$ 5.4 for cisplatin *vs* IC$_{50}$ = 1.7 $\pm$ 0.7 for **6**). This result is particularly relevant since SK-MEL-28 malignant melanoma cell line is notoriously resistant to many chemotherapeutic drugs, including cisplatin.[32] Hence, conjugation of the Pt(IV) pro-drug to the tetrameric RAFT-RGD results in a compound with much higher antitumor activity than its parent Pt(II) complex or cisplatin. In addition, comparison of the cytotoxic activity of conjugates **5** and **6** in SK-MEL-28 cells demonstrates that four copies of the RGD-containing motif assembled on the RAFT cyclodecapeptide scaffold are preferred over a single copy (IC$_{50}$ = 12.8 $\pm$ 2.1 *vs* IC$_{50}$ = 1.7 $\pm$ 0.7, respectively) from a chemotherapeutic drug efficacy point of view. These effects were not observed in CAPAN-1 or 1BR3G cells, selected as negative control based on the low the expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins. In these cell lines, the conjugation of the Pt(IV) derivative of picoplatin to the carrier peptides resulted in a marked reduction of the cytotoxic activity when compared with picoplatin. IC$_{50}$ values for **5** were over 50 μM in both cell lines whereas the IC$_{50}$ value for **6** was also over 50 μM in 1BR3G cells and reduced by half in CAPAN-1 cells (IC$_{50}$ of 27.2 $\pm$ 5.7). The fact that

the antitumor activity of the conjugates was inhibited in the absence of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin expression supports the suitability of using RGD-containing peptides, particularly a multimeric version of this scaffold, to selectively deliver metallodrugs in cancer cells.

Table 1. Sensitivity of SK-MEL-28, CAPAN-1 and 1BR3G cells to cisplatin, picoplatin, conjugates **5**-**6** and control peptides.

Compound	IC$_{50}$ (μM)[a]		
	Cell line		
	SK-MEL-28	CAPAN-1	1BR3G
Cisplatin	17.0 ± 5.4	2.25 ± 0.3	3.9 ± 0.2
Picoplatin	33.6 ± 6.6	13.8 ± 1.0	23.2 ± 3.5
Pt-c(RGDfK) **5**	12.8 ± 2.1	> 50	> 50
Pt-RAFT-{c(RGDfK)}$_4$ **6**	1.7 ± 0.6	27.2 ± 5.7	> 50
c(RGDfK) **8**	18.5 ± 5.5	> 50	> 50
RAFT-{c(RGDfK)}$_4$ **11**	2.1 ± 0.7	> 50	> 50

[a] The concentration of the compounds that inhibits cells viability by 50% (IC$_{50}$) after 72 h was determined by means of the MTT assay. Each value represents the mean of three independent experiments $\pm$ SD.

MTT assays also revealed a marked effect of the unconjugated peptides on SK-MEL-28 cell viability. It is well described that RGD-containing peptides and derivatives can display antiproliferative properties by interacting with the cell surface integrins, which leads to the cells detachment from the extracellular matrix and conformational changes that induce an anchorage dependent apoptosis, named anoikis.[33] We observed that exposure of the cells to the unconjugated peptides at 10 μM induced morphologic changes followed by cell detachment from the plate surface. Changes were greater in cells exposed to the tetrameric peptide (RAFT-{c(RGDfK)}$_4$, **11**) compared to the monomeric version (c(RGDfK), **8**) (Fig. S15, see the Supporting Information). Similar effects on the cell morphology and adhesion were detected for the unconjugated RAFT-{c(RGDfK)}$_4$ and for its platinum conjugate, Pt-RAFT-{c(RGDfK)}$_4$ at 5 μM, while no changes on the cell

morphology were observed after picoplatin treatment. Thus, unconjugated RGD-containing peptides seem to exert some antiproliferative effects in SK-MEL-28 cells by disrupting the integrin mediated cell adhesion. In this sense, the tetrameric RAFT-RGD peptide, with higher binding affinity for integrins than the monomeric RGD peptide, showed the highest antiproliferative activity (IC_{50} of 2.1 $\pm$ 0.8 μM for **11** *vs* 18.5 $\pm$ 5.5 μM for **8**). Our results are in accordance with a previous report describing pro-apoptotic activity of RGD-analogues in melanoma cells, although they describe that the biological effects may be independent from their antiadhesive properties.[34] Notably, the concentration-response curves, plotted in Fig. 5, revealed that the cytotoxic activities of both Pt-peptide conjugates were higher than those determined for the corresponding unconjugated peptides, although only small differences were detected between Pt-RAFT-{c(RGDfK)}$_4$ and RAFT-{c(RGDfK)}$_4$. These results seem to indicate that in addition to the proapoptotic effects of the peptides, the Pt(IV) pro-drug contributes to the antitumor activity of the conjugates.
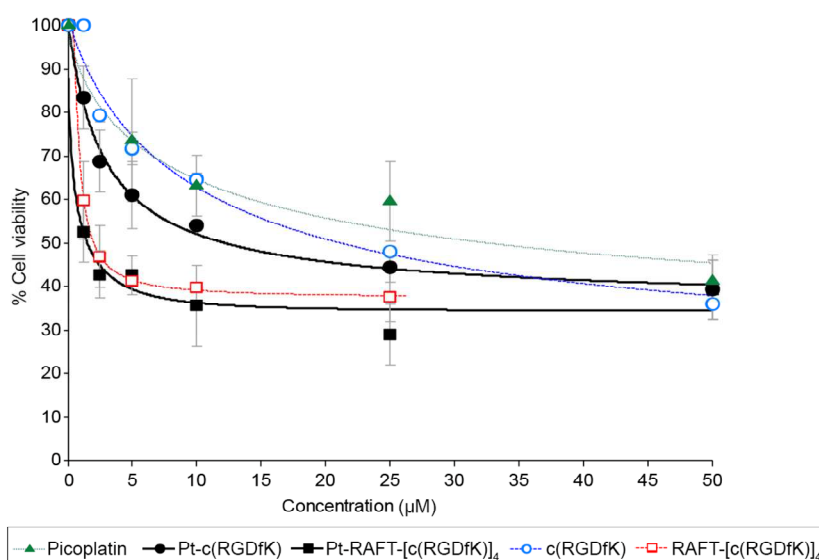
Fig. 5 Cytotoxic effect of picoplatin, conjugates **5-6** and control peptides in the SK-MEL-28 cells. Cells were treated for 72 h with the indicated concentrations of each compound. Cell viability was determined by the MTT assay. Each point in the graphs represents the mean of three independent experiments $\pm$ SD.

Intracellular uptake of Pt-peptide conjugates by ICP-MS.

Since metal accumulation into cells is essential for the cytotoxicity of metal-based anticancer drugs, the investigation of the cellular uptake efficiency is of high relevance to determine the potential activity of the compounds as well as to understand their mechanism of action. This is particularly relevant when using biological carrier compounds,[35] such as peptides whose receptors are overexpressed on the membrane of cancer cells, since the uptake of the metal-peptide conjugate might be conditioned by the level of expression of the receptors and by the binding affinity between the resulting conjugate and the target receptor. In our case, according to internalization studies with fluorescein-labeled peptides **12** and **13**, the internalization of Pt-RAFT-{c(RGDfK)}$_4$ was expected to be much higher than that of the monomeric conjugate Pt-c(RGDfK), which is in good agreement with its higher cytotoxicity, attributable to a greater intracellular Pt accumulation. However, the proapoptotic effects of the unconjugated peptides, particularly of the tetrameric RAFT-{c(RGDfK)}$_4$, led us to investigate the cellular uptake of the two Pt(IV)-peptide conjugates, Pt-c(RGDfK) (**5**) and Pt-RAFT-{c(RGDfK)}$_4$ (**6**) in the melanoma cancer cell line, as well as that of the parent platinum(II) complex, picoplatin. The pancreas adenocarcinoma cell line was also included in the study as negative control for integrin expression. Intracellular levels of platinum in both cancer cell lines were quantified by inductively-coupled plasma mass spectrometry (ICP-MS) using ^{196}Pt detection[36] after a 24 h exposure to the compounds at the same concentration (0.5 µM), which was in all cases below their IC$_{50}$ values in both cell lines.

As shown in Fig. 6, the accumulation of platinum (determined as the net effect of influx and efflux of Pt) after exposure of SK-MEL-18 cells to Pt-c(RGDfK) conjugate (**5**) (12.64 $\pm$ 1.13 pmol Pt/10^6 cells) was higher than that of the parent picoplatin complex (8.44 $\pm$ 1.53 pmol Pt/10^6 cells), which indicates that the RGD-containing peptide has a positive effect on the intracellular accumulation of the Pt(IV) pro-drug. Interestingly, the tetrameric conjugate, Pt-RAFT-{(cRGDfK)}$_4$, was accumulated in the melanoma cells (22.41 $\pm$ 0.07 pmol Pt/10^6 cells) at a much higher amount than the parent Pt(II) complex (about 2.7-fold) or the monomeric conjugate (about 1.8-fold). Such a significant differences between these compounds highlight again the potential of multivalency in

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targeted anticancer strategies since conjugation of the Pt(IV) complex to the RAFT cyclodecapeptide moiety containing four copies of the RGD motif results in higher accumulation than when the pro-drug was conjugated to a single RGD-containing peptide. These results are also in good agreement with our internalization experiments by flow cytometry with peptides **12** and **13** as well as with those previously reported in the literature with similar RGD-containing macroestructures, and indicate that the capacity of integrin-mediated internalization of RGD peptides was not substantially modified by the attachment of the metallodrug.
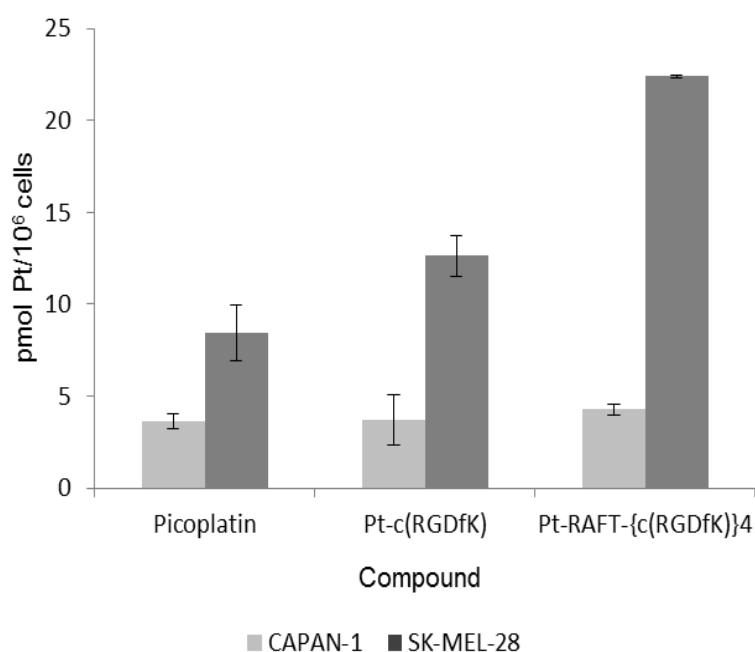
Fig. 6 Cell accumulation of platinum in SK-MEL-28 and CAPAN-1 cells after exposure to picoplatin and conjugates **5** and **6** (0.5 μM, 24 h). The platinum content is related to the cell number. Errors bars represent the standard deviation of three replicates

Platinum accumulation in CAPAN-1 cells after exposure to conjugates **5** (3.69 ± 1.37 pmol Pt/10^6 cells) and **6** (4.27 ± 0.29 pmol Pt/10^6 cells) was similar and substantially lower than in SK-MEL-28 cells (about 3.4-fold and 5.2-fold, respectively). This result agrees with the internalization studies and with the lower level of expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins in CAPAN-1 cells compared with SK-MEL-28 cells, and confirms the selectivity of RGD-containing peptides, particularly the tetrameric RAFT-{c(RGDfK)}$_4$ peptide, against cells overexpressing these receptors.

It is worth highlighting the existence of a clear correlation between the internalization and accumulation of the conjugates (Figs. 4 and 6) and their antitumor activity (Table 1 and Fig. 5). Effectively, the cytotoxic activity of conjugates **5** and **6** was much higher in SK-MEL-28 cells than in CAPAN-1 cells, and cytotoxicity of the tetrameric conjugate was even higher than that of the monomeric conjugate. This indicates that the great ability of RAFT-{c(RGDfK)}$_4$ to bind to and to be internalized by $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins overexpressed in SK-MEL-28 results in greater accumulation of the Pt(IV) complex when conjugated, thereby leading to a higher antitumor efficacy. By contrast, the lower expression of integrins in CAPAN-1 cells hinders the internalization of the Pt(IV) pro-drug conjugated to the RGD-containing peptides and, as a consequence, a reduced platinum accumulation leads to lower or even null cytotoxic activity. Surprisingly, this correlation was not found in the case of the parent platinum(II) complex. Indeed, despite the higher accumulation of picoplatin in SK-MEL-28 cells (8.44 ± 1.53 pmol Pt/10^6 cells) compared with CAPAN-1 (3.65 ± 1.53 pmol Pt/10^6 cells), the antitumor efficacy was about 2.4-fold lower in the melanoma cancer cell line than in the pancreas cancer cell line (e.g. IC$_{50}$ = 33.6 ± 6.6 in SKMEL-28 *vs* IC$_{50}$ = 13.8 ± 1.0 in CAPAN-1), which agrees with the known resistance of SK-MEL-28 cell line to Pt-based drugs.

Activation by reduction of the Pt-peptide conjugates

As previously stated, the greater inertness of Pt(IV) complexes in blood compared with their Pt(II) precursors makes this family of compounds very attractive as chemotherapeutic agents with the aim of reducing toxic side-effects of Pt(II) metallodrugs. In addition, the functionalization of the axial positions of the Pt(IV) complex with carrier molecules offers the possibility to target cancer cells in a selective manner. Despite these advantages, after internalization and accumulation, Pt(IV) pro-drugs need to be intracellularly activated by reduction to generate the cytotoxic Pt(II) species. In the case of functionalizing one or two axial positions of the Pt(IV) complex with a carrier, such as in the Pt-peptide conjugates **5** and **6** reported in this work, the release of the carrier will occur during the reduction step. Among possible biological reducing agents, ascorbate, glutathione and some cysteine-containing proteins have been postulated to participate in the activation of Pt(IV)

complexes.[20a,21b,37] In order to gain some insight on the capacity of the Pt(IV) derivative of picoplatin to be activated by reduction when conjugated to RGD-containing peptides, we selected ascorbate as reducing agent. For this purpose, Pt-c(RGDfK) conjugate (**5**) was allowed to react in water (0.25 mM) with an excess of ascorbate (5 mol equiv.). 5'-Guanosine monophosphate (5 mol equiv.) was also added to act as a simple model of DNA for capturing the Pt(II) species generated upon reduction (Scheme 3).
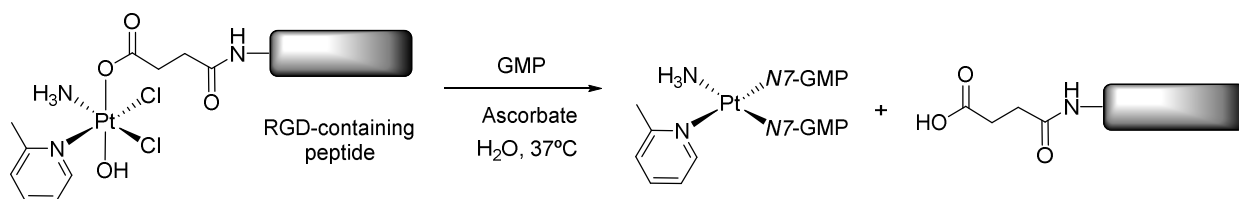
Scheme 3. Reaction between Pt-peptide conjugates and 5'-GMP mediated by ascorbate reduction.

The monitoring of the reduction process was carried out by reversed-phase HPLC coupled to a ESI mass spectrometer. As shown in Figs. S16-S18 (see the Supporting Information), the peak corresponding to conjugate **5** (R_t = 9.9 min) disappeared completely after incubation at 37ºC for 24 h, while some new peaks appeared, including a major peak at R_t = 6.7 min. Most of those intermediate peaks evolved into that major peak after 72 h. This compound was characterized by MS as the adduct between the platinum(II) moiety $\{Pt(NH_3)(2\text{-pic})\}^{2+}$ and two GMP. The fact that the maximum wavelength absorption of *cis*-$[Pt(GMP)_2(NH_3)(2\text{-pic})]^{2+}$ (the charge on the GMP is ignored in the formula) was shifted by 4 nm (259 nm) with respect that of GMP (255 nm) indicates coordination of platinum to the *N7* of guanosine. The release of the succinate derivative of the peptide (R_t = 9.5 min) was also confirmed by MS.

The overall results indicate that the Pt(IV) derivative of picoplatin conjugated to RGD-containing peptides can be activated by typical intracellular reducing agents leading to an activated Pt(II) species with capacity to react with DNA nucleobases, as inferred by the main adduct generated upon reaction with GMP. This indicates that the covalent attachment of the RGD-containing peptide to the Pt(IV) complex does not seem to interfere with the reduction process. Hence, we can envisage from cellular uptake studies by ICP-MS in SK-MEL-28 cells that the intact Pt-peptide conjugates are internalized

and accumulated in the cancer cell, where they are expected to be activated by reduction to generate their antitumor effect. Otherwise, a premature reduction of the Pt(IV) complex conjugated to the peptides prior to the integrin-mediated internalization would lead to similar or even lower Pt accumulation ratios than those obtained with the reference complex, picoplatin. Although we have observed a participation of the RGD-containing peptides in the cytotoxic activity of the conjugates, particularly that of the tetrameric RAFT-{c(RGDfK)}$_4$ peptide in the melanoma cancer cell line, the overall results seem to point out to an important contribution of the Pt(IV) complex derived from picoplatin in the antitumor activity of the Pt-peptide conjugates.

CONCLUSIONS

In summary, in this work we have explored the use of RGD-containing peptides, particularly a RAFT-{c(RGDfK)}$_4$ derivative that contains four copies of the RGD motif, to deliver Pt(IV) anticancer metallodrugs into tumor cells overexpressing $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins in a selective manner. By using solid- and solution-phase approaches, a Pt(IV) derivative of *cis*-ammine(2-methylpyridine)dichloridoplatinum(II) (picoplatin) was conjugated to monomeric (Pt-c(RGDfK), **5**) and tetrameric (Pt-RAFT-{c(RGDfK)}$_4$, **6**) RGD-containing peptides. In order to evaluate the biological activity of the compounds, the SK-MEL-28 malignant melanoma cell line was selected on the basis of a high expression level of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins. The use of the corresponding fluorescein-labeled peptides revealed a good correlation between integrin expression and their cellular uptake and, more importantly, that the internalization capacity of the tetrameric RGD-containing peptide was considerably higher than that of the monomeric one. As expected, cellular uptake was substantially reduced in CAPAN-1 pancreatic cancer cells and 1BR3G fibroblasts, which were selected as negative control due to a very low $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin expression. Interestingly, a good correlation was found between the cytotoxic activity of the conjugates and their intracellular accumulation, which was determined by ICP-MS. On the one hand, the antitumor efficacy of picoplatin in SK-MEL-28 cells was increased by 2.6-fold when its Pt(IV) derivative was conjugated to c(RGDfK) (IC$_{50}$ = 12.8 $\pm$ 2.1 µM) and, more importantly, by 20-fold when conjugated to RAFT-

{c(RGDfK)}$_4$ (IC$_{50}$ = 1.7 $\pm$ 0.6 µM). However, the cytotoxicity of the conjugates was inhibited in CAPAN-1 and 1BR3G cells lacking $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin expression. On the other hand, cellular uptake studies after exposure to both conjugates indicated that platinum accumulation was much higher in SK-MEL-28 cells than in CAPAN-1, being particularly higher in the case of the tetrameric conjugate (**6**) compared with that of the monomeric one (**5**). The overall results highlight the great capacity of RAFT-{c(RGDfK)}$_4$ to bind to and to be internalized by integrins overexpressed in SK-MEL-28 cells, which results in higher intracellular accumulation of the platinum pro-drug and, consequently, in a higher antitumor activity. Importantly, the Pt(IV) pro-drug can be activated by typical intracellular reducing agents such as ascorbate even when conjugated to the peptide to generate the cytotoxic Pt(II) species. To our surprise, the tetrameric RAFT-RGD peptide showed an intrinsic antiproliferative activity in the melanoma cancer cell line. Although the overall results seem to indicate that the Pt(IV) derivative of picoplatin contritubes to the antitumor activity of the Pt-peptide conjugates, further studies will be necessary to evaluate the impact of this multivalent RGD-containing peptide in other cancer cell lines overexpressing $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins.

These studies provide new insights into the potential of exploiting specific alterations found in human tumor cells, such as the overexpression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins, to develop new chemotherapeutic drugs with reduced side-effects and reduced toxicity in normal cells. The potential of this receptor-targeted anticancer strategy is expected to be increased by conjugating photoactivatable Pt(IV) anticancer pro-drugs[38] to tumor-targeting devices based on RGD-containing sequences, particularly on multivalent derivatives, or other receptor-binding peptides, with the aim of improving their selectivity and potency by increasing intracellular accumulation in tumor tissues. Work is in progress in this direction.

ACKNOWLEDGEMENTS

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