Solid-phase synthesis of platinum peptide conjugates for targeted drug delivery*

Abstract - Mononuclear and dinuclear platinum complex tethered to an $\alpha_9\beta_1$-integrin-targeting peptide and a nuclear-localisation peptide have been synthesised using solid-phase synthesis. The cellular uptake, DNA binding and cytotoxicity of the complexes was monitored in a number of different cell lines. These results show that some of the desired selectivity is obtained for both targeting systems.

4.1 INTRODUCTION

Although chemotherapy has been used in oncology for more than a century, cancer remains a major cause of death [1]. Therefore an urgent and continuous need exists for more effective chemotherapeutics. The inorganic drug cisplatin is highly efficient in the treatment of a number of solid malignancies through triggering apoptotic pathways in the tumour cells [2]. The application of cisplatin is, however, hampered by systemic toxicity, with dose-limiting side-effects including neuro- and nephrotoxicity. In addition, the occurrence of drug resistance limits the use of cisplatin in the clinic [3]. Cisplatin resistance can be overcome to some degree by polynuclear platinum complexes [4-6], but toxicity remains a problem. An important strategy towards reducing systemic toxicity is to improve delivery of the platinum drugs to their target. The therapeutic effect of all platinum drugs is ascribed to the involvement in the generation of multiple lesions on the DNA of cancerous cells [7]. Targeting strategies can therefore be envisaged that involve either selective delivery to cancer cells, or improved delivery to nuclear DNA. Many literature examples exist that describe platinum drugs tethered to targeting ligands to regulate the uptake of the drug or improve the affinity for DNA, as described extensively in Chapter 2 of this thesis [8]. In particular macromolecular constructs or formulations that are taken up selectively in tumours due to the enhanced permeation and retention (EPR) effect [9] have proved successful in early clinical evaluations. With the wealth of synthetic membrane-active peptides available in literature [10], a generalised method for the synthesis of platinum-peptide conjugates could facilitate the discovery of new chemotherapeutics targeted towards specific tissue or to DNA, ultimately leading to the development of new targeted therapies.

In Chapter 3 of this thesis a convenient solid-phase synthetic method for the preparation of dinuclear peptide platinum conjugates is described [11]. In combination with a method developed for the synthesis of mononuclear platinum peptide conjugates [12,13], previously used for studying the effect of short appended peptides on platinum-DNA binding [14], this
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Method can be used to link different platinum drug moieties to peptide transporter molecules, known to shuttle drugs past physiological barriers. For the present study two peptides were selected from the numerous peptide sequences described as vector ligands in literature, i.e. a peptide which targets specific tissue and a peptide targeting nuclear DNA in general [10]. The GPLAEIDGIELG domain peptide (GPL) was chosen to selectively target \( \alpha_9\beta_1 \)-integrin-displaying cells. It has been shown that peptide-DNA conjugates containing this sequence can transfect such cells via receptor-mediated endocytosis [15]. The \( \alpha_9\beta_1 \)-integrin receptor is only displayed in a few highly specialised tissues and has been associated with certain colon cancers [16], thereby presenting a potential target for cancer therapy. The nuclear localisation signalling (NLS) peptide from the HIV-1 matrix protein (GKKQYKLKH) was selected for targeting the nucleus of all cells. NLS peptides direct proteins into the nucleus through binding to the nuclear import receptor subunit karyopherin \( \alpha \) [17]. Based on the GPL and NLS peptides, mono- and dinuclear platinum complexes were synthesised, yielding a total of four targeted platinum complexes (1-4, Figure 4.1).

Figure 4.1: Two mononuclear (1, 3) and two dinuclear (2, 4) peptide targeted platinum complexes, with targeting peptide being either GPLAEIDGIELG (GPL) or GKKQYKLKH (NLS).

Monitoring the efficacy of peptide-based targeting ligands in cell cultures can be achieved using different methods. A fluorescent label may be attached to the peptides at either the C-terminus, or to a side-chain of one of the amino acids. Such an approach has been used to study the behaviour of platinum drugs inside the cell [18-20]. The disadvantage of this technique, however, is that by attaching the label, the physical and chemical properties of the complexes may be changed significantly, and generalisations made about the labelled complexes may not necessarily hold for their unlabelled counterparts. It was therefore decided to use two alternative approaches in the current study. Firstly, both the platinum content inside the cell and the platinum bound to DNA was quantified using ICP-OES. For this the cells need to be destroyed prior to measurement. Consequently, a time-dependant study is
more difficult to perform. Furthermore it is not possible to distinguish between the different cellular compartments. As an advantage however, the cell uptake of platinum complexes can be measured directly without modification of the complexes studied. Secondly, a cytotoxic assay was performed. This gives indirect insight to the transport of the complexes: if a complex displays toxicity, it must have entered the cell. Furthermore, a cytotoxicity assay provides an indication as to whether a complex may be a promising drug candidate.

4.2 PREPARATION OF THE COMPLEXES

The synthesis of the complexes is illustrated in scheme 4.1. The targeting peptides were assembled on a peptide synthesiser, using a standard Fmoc protocol [21]. During the last coupling cycle either N-(2-aminoethyl)-N,N’-di(9-fluorenylmethyloxycarbonyl)glycine or N-α,ε-di(9-fluorenylmethyloxycarbonyl)-L-lysine was coupled to the resin-bound peptides followed by selective deprotection using piperidine (20% in NMP), yielding the immobilised peptides 7-10. The integrity of the peptides was verified by treating a small portion of resin with 95% TFA in water and analysing the released and unprotected peptides by LC-MS. Invariably this revealed a peak with the desired m/z of high purity, greater than 95%.

Scheme 4.1: Solid-phase synthesis of peptide targeted platinum complexes. a) N-(2-aminoethyl)-N,N’-di-Fmoc-glycine, HCTU (1 equiv), DIPEA (2 equiv), then 20% pip. b) N-α,ε-di-Fmoc-L-lysine, HCTU (1 equiv), DIPEA (2 equiv), then 20% pip. c) 5 equiv of K₂PtCl₄ in water/NMP (1/9). d) 5 equiv of trans-diamminedichloroplatinum(II) activated by overnight reaction with AgNO₃ in NMP.
In the following step, platinum was coordinated to the peptides. For peptides 7 and 9 platination was effected by treatment with 5 equiv of K₂PtCl₄ in water/NMP (1/9) to yield the mononuclear compounds 11 and 13. For peptides 8 and 10 5 equiv of trans-diamminedichloroplatinum(II), activated by overnight reaction with AgNO₃ in NMP, was used to yield the resin-bound dinuclear complexes 12 and 14. TFA (95% in water) was used to release the complexes from the resin and remove the protective groups present at the side chains of the amino-acid residues. Subsequent diethyl ether precipitation afforded the desired complexes 1-4 as off-white solids. Preparative gel filtration on an SW40 column (1% AcOH, 20% MeOH in H₂O for 1, 3, 4 and 50% MeOH for 2) was used for purification of the complexes. The final yield after purification ranged between 20 and 40%.

When analysing the purified products using HPLC, no unplatinated starting material was observed, and the products appeared as a broad peak at 5-10 min longer retention times compared to the starting peptides. The ¹⁹⁵Pt NMR spectra of the four complexes reveal the expected signals around -2350 ppm for the [PtN₂Cl₂] chromophore present in compounds 1 and 3 and the [PtN₃Cl] chromophore of compounds 2 and 4. ICP-OES was used to accurately determine the platinum concentration in a standard solution of 60 µM of the compounds, confirming the purity of the complexes. It proved to be difficult to obtain MS-spectra for these compounds; however, useful results were obtained using MALDI-TOF MS. Although all spectra contain a high degree of noise, for complexes 1 and 3 signals for (M-2Cl⁻) accompanied by signals of the unplatinated peptide are observed. Similarly the spectra of the dinuclear compound 4 shows signals for (M-2Cl⁻) and the unplatinated peptide. For compound 2 only the unplatinated peptide is observed.

4.3 BIOLOGICAL EVALUATION OF INTEGRIN-TARGETED COMPLEXES

For the study of the GPL complexes 1 and 2 an sw480 colon carcinoma cell line, displaying and lacking the α₉β₁-integrin receptor was used [15]. As these two cell lines are otherwise identical, differences observed can be related directly to the integrin receptor. For the uptake experiments cells were treated with the platinum complexes 1, 2 and cisplatin at a concentration of 50 µmol for 2h, after which the cells were washed, lysed and the platinum content was determined using ICP-OES. The results are presented in table 4.1. Cisplatin and compound 2 are taken up equally well in both cell lines. Surprisingly, compound 1 appears to be taken up slightly better in the sw480⁻ cells compared to the sw480⁺ cells. In contrast, the IC₅₀ values, defined as the concentration of compound needed to inhibit 50% of cell growth, obtained for compound 1 and 2 in the cell line displaying the receptor are clearly lower than the IC₅₀ values for the compounds in the cell line lacking the receptor. Cisplatin conversely
shows higher activity in the cell line lacking the receptor. This difference indicates that a degree of the desired selectivity is obtained for the platinum peptide conjugates. Unfortunately for all compounds a clear effect on cell-proliferation in both cell lines is observed. Therefore, it cannot be concluded that uptake of these complexes occurs exclusively via the \( \alpha_9\beta_1 \)-integrin receptor, and other uptake mechanisms may play an important role in the cellular trafficking of these complexes.

<table>
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<tr>
<th>Table 4.1: Cellular uptake and IC(_{50}) values for compounds 1, 2 and cisplatin in the human colon carcinoma cell line sw480 displaying (sw480(^+)) or lacking (sw480(^-)) the ( \alpha_9\beta_1 )-integrin receptor.</th>
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### 4.4 BIOLOGICAL EVALUATION OF DNA-TARGETED COMPLEXES

For complexes 3 and 4 uptake studies were performed on the A2780 ovarian carcinoma cell line (table 4.2). This is one of the most commonly used cell lines for the evaluation of platinum antitumour compounds [22]. To quantify the effect of the NLS peptide on the complexes, both cellular uptake and DNA binding were measured. For both experiments cells were incubated with compounds 3, 4 and cisplatin at 50 \(\mu\)M for 2 h. Once the cells were lysed and collected, the cellular uptake could be measured directly. For DNA-binding studies, DNA was isolated, redissolved in water and the concentration of platinum in the resultant solution was determined.

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<th>Table 4.2: Cellular uptake, DNA binding and IC(_{50}) values for compounds 3, 4 and cisplatin in the human ovarian carcinoma cell line A2780.</th>
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For compounds 3 and 4 a slight increase in intracellular platinum concentration compared to cisplatin is observed, however this increase is not statistically relevant. The level of DNA platination is, furthermore, not increased by the presence of the NLS peptide. The peptide therefore does not show the desired targeting effect on the platinum drug moieties. Compounds 3 and 4 are 8-10 fold less cytotoxic than cisplatin. Any effect of the peptide is, therefore, clearly not to increase the efficacy of the complexes.

4.5 CONCLUSION

In this study a peptide targeting specific tissue, as well as a peptide targeting nuclear DNA, was linked to both mono- and dinuclear platinum moieties using solid-phase synthesis to obtain four targeted platinum complexes.

The GPL complexes 1 and 2, targeted to cells displaying the α9β1-integrin receptor, showed an increased cytotoxic effect in the sw480+ cells relative to the sw480− cells. However, the cellular uptake and the cytotoxic effect in cells lacking the receptor was such that uptake of these complexes most likely also occurs via pathways other than endocytosis via the α9β1-integrin receptor. Fine-tuning the selective uptake of these complexes may therefore lead to more useful targeted platinum drugs. The GPL peptide makes the complexes rather hydrophobic, which possibly favours passive diffusion across the cell membrane. To improve the targeting of these complexes, a hydrophilic spacer, such as poly-K, could be introduced on either the N or the C-terminus of the GPL peptide. This would make the passive uptake mechanism less-favoured, thereby promoting selective uptake via the α9β1-integrin receptor.

Unlike the GPL-complexes the NLS-complexes are highly polar and show extremely high solubility in water. The uptake of the complexes into the cell may be increased compared to the untargeted cisplatin. However, this uptake neither leads to a higher degree of DNA platination, nor to an increased cytotoxic effect compared to cisplatin. In this case there may be interaction between the peptide and the platinum part of the molecules impairing with each others’ function. It is possible that through introduction of a spacer between the functional peptide and the platinum-drug this interaction can be decreased, yielding complexes displaying a more favourable biological behaviour.
4.6 EXPERIMENTAL SECTION

4.6.1 General
Chemicals and solvents were purchased from Acros, Nova-Biochem and Biosolve and used as received unless otherwise stated. The trans-[Pt(NH₃)₂Cl₂] was obtained using literature procedures, from K₂PtCl₄. NMR measurements were performed on a 300 MHz Bruker DPX300 spectrometer with a 5 mm multi-nucleus probe. Temperature was kept constant at 298 K, using a variable temperature unit. ¹⁹⁵Pt chemical shifts were referenced to Na₂PtCl₄ (δ=0 ppm). MALDI-TOF MS was performed on an Applied Biosystems Voyager using a 4-hydroxy-α-cyano cinnamic acid matrix. Platinum ICP-OES was determined in ppb at 214.424 nm on a VISTA-MPX spectrometer. LC-MS was measured with an Alltima 3µ C18 reversed phase column (150 x 4.6 mm) using a ThermoFinnegan AQA ESI-MS for mass analysis. The eluent used was H₂O:ACN + 1% TFA in a gradient of 5-70% ACN over 35 min.

4.6.2 Peptide synthesis
Peptide synthesis was accomplished on a ABI-peptide synthesiser using Rink amide HMBA resin [23] at a loading of approximately 0.25 mmol/g. During each coupling cycle a solution of Fmoc-protected amino acid (5 equiv), HCTU (5 equiv) and DIPEA (10 equiv) in NMP was reacted with the resin for 1 h. After washing (NMP, DCM) the resin was treated with 20% piperidine in NMP (5 × 1 min) and washed (NMP, DCM) to complete the cycle. Commercially available Fmoc-protected amino acids were used with functional groups in the side chain, protected by acid-labile groups Boc (K), tBu (E, D, Y) or Tr (Q, H). For LC-MS analysis a small portion of resin (<5 mg) was treated with 95% TFA in water for 2 h, after which the solution was injected directly onto the HPLC.

5: LC-MS m/z (calc): 1182.9 (1183.3) [M+H]^+. 6: LC-MS m/z (calc): 1128.8 (1127.7) [M+H]^+, 565.2 [M+H]^2+. 7: LC-MS m/z (calc): 1282.8 (1282.4) [M+H]^+. 8: LC-MS m/z (calc): 1310.9 (1311.5) [M+H]^+. 9: LC-MS m/z (calc): 1228.8 (1227.8) [M+H]^+. 10: LC-MS m/z (calc): 1256.0 (1255.8) [M+H]^+.

4.6.3 Mononuclear complex synthesis
K₂PtCl₄ (108 mg, 0.26 mmol) was dissolved in water:NMP (1:9) to a concentration of 0.05 M and added to resin-bound peptides 7 or 9 (100 mg, 0.026 mmol) and shaken overnight in the dark to yield 11 and 13. The target material was cleaved from the resin by treatment with 1 ml of 95% TFA in H₂O for 2 h. The solution was drained into 10 ml of diethyl ether and a precipitate formed instantly. An additional 1 ml of TFA solution was added to the resin and drained into the diethyl ether for washing of the resin. After 1 h at -20 °C the product was centrifuged, washed with diethyl ether (3 ×), redissolved in H₂O:ACN (1:1), filtered and lyophilised to give the products 1 and 3 as off-white powders. Purification was effected by
preparative gel filtration on an SW40 column (1% AcOH H₂O in 20% MeOH). ICP was measured on 3 ml of 60 µM solution in 20% HNO₃ (aq).

1: [PtCl₂(edaGPLAEIDGIELG)]: ¹⁹⁵Pt NMR (D₂O/ACN-d₃, 1/1) δ (ppm): -2357. Calculated Mass: 1548.4. MALDI-TOF MS m/z: 1477.6 [M-2Cl], 1283.4 [M-PtCl₂]. ICP (calc): 3930 ppb (3900 ppb). Yield: 31%.

3: [PtCl₂(edaGKKQYKLKH)]: ¹⁹⁵Pt NMR (D₂O/ACN-d₃, 1/1) δ (ppm): -2354 ppm. Calculated Mass: 1492.4. MALDI-TOF MS m/z: 1423.2 [M-2Cl], 1228.1 [M-PtCl₂]. ICP (calc): 3872 ppb (3900 ppb). Yield: 21%.

4.6.4 Dinuclear complex synthesis

trans-[Pt(NH₃)₂Cl₂] (78 mg, 0.26 mmol) was activated by treatment with AgNO₃ (44 mg, 0.25 mmol) in DMF (1.5 mL) overnight in the dark. AgCl was removed by filtration. The transplatin solution and TEA (0.35 mmol, 7 equiv) was added to preswollen resin 8 or 10 (100 mg, 0.026 mmol) and the mixture was shaken overnight in the dark to yield 12 and 14. The target material was cleaved from the resin by treatment with 1 ml of 95% TFA in H₂O for 2 h. The solution was drained into 10 ml of diethyl ether and a precipitate formed instantly. An additional 1 ml of TFA solution was added to the resin and drained into the diethyl ether for washing of the resin. After 1 h at -20 °C the product was centrifuged, washed with diethyl ether (3 ×), redissolved in H₂O:ACN (1:1), filtered and lyophilised to give the products 2 and 4 as off-white powders. Purification was effected by preparative gel filtration on an SW40 column (1% AcOH H₂O in 20% MeOH for 4 and 50% MeOH for 2). ICP was measured on 3 ml of 60 µM solution in 20% HNO₃ (aq).

2: [(Pt(NH₃)₂Cl)₂KGPLAEIDGIELG]: ¹⁹⁵Pt NMR (DMF) δ (ppm): -2379 ppm. Calculated Mass: 1839.7. MALDI-TOF MS m/z: 1311.9 [M-2Pt(NH₃)₂Cl]. ICP (calc): 7734 ppb (7800 ppb). Yield: 37%.

4: [(Pt(NH₃)₂Cl)₂KGKKQYKLKH]: ¹⁹⁵Pt NMR (DMF) δ (ppm): -2331 ppm. Calculated Mass: 1785.7. MALDI-TOF MS m/z: 1714.5 [M-2Cl], 1256.7 [M-2Pt(NH₃)₂Cl]. ICP (calc): 7320 ppb (7800 ppb). Yield 29%.

4.6.5 In vitro studies

The sw480 cell were generously donated by Dr. D. Sheppard (School of Medicine, University of California, San Francisco). The A2780 cells were used as a generous gift from Dr. J.M. Perez (Universidad Autonoma de Madrid, Spain). Cells were grown as monolayers in Dulbecco’s modified Eagle’s Medium supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland), penicillin (100 units/ml: Dufecha, Netherlands) and streptomycin (100 µg/ml: Dufecha, Netherlands). For the sw480⁺ cells, medium was supplemented with G418 (200 µg/ml).
For cellular uptake studies cells were grown in 12-well plates until the wells were approximately 80% filled. Compounds 1-4 were added in triplicate to the wells to reach a total concentration of 50 µmol and they were left to incubate for 2 h. The solution was removed and the cells were washed twice with PBS. The cells were then treated with lysis buffer (SDS 5% H₄EDTA, 0.8 ml). For determination of whole-cell platinum uptake the volume was brought to 2.5 ml using PBS and the samples were sonicated for 4 h after which the platinum content was measured using ICP-OES. For determination of the DNA binding, DNA was isolated from the lysed cell fractions and redissolved in a 20% HNO₃ solution (2.5 ml) and the platinum content was measured. All experiments were repeated once.

For the IC₅₀ determination cells were pre-cultured for 48 h at 37 °C in a 7% CO₂ containing incubator in 96 multi-well plates and subsequently treated with 100 µl of compound at six different concentrations from 50 to 0 µmol in quadruplicate. After 72 h, MTT in PBS (100 µl at 2.5 mg/ml) was added and the cells were incubated for 2 h. The solution was carefully removed and the remaining crystals dissolved in 100 µl of DMSO after which the absorbance at 590 nm of each well was determined using a plate reader. The growth inhibition was determined relative to untreated controls. The experiments were repeated three times.
REFERENCES

Chapter 4
