4.

Relevance of the leaving group for the antitumor activity of new platinum(II) compounds containing E-1-(9-anthryl)-3-(2-pyridyl)-2-propenone, A9pyp, as a carrier ligand*

Abstract

The relevance of the leaving groups for the biological activity of cis-[Pt(A9pyp)(dmso)Cl2] (2) and [Pt(A9pyp)(dmso)(cbdca)] (3), in which A9pyp is E-1-(9-anthryl)-3-(2-pyridyl)-2-propenone, has been investigated. It is known that hydrogen carbonate ions present in cells and extracellular fluids play an important role towards ring-opening of the carboxylate chelating ring in carboplatin, affecting its hydrolysis rate. These observations address the study of compound 3 in the presence of hydrogen carbonate, to investigate the ring-opening of the cbdca chelating ligand and its hydrolysis behavior compared to the reaction of compound 2 with the hydrogen carbonate anion. Different reaction rates with carbonate species have been found between compounds 2 and 3 a most unusual, unprecedented water addition to the double bond in the ligand A9pyp has been found, and where 2 reacts faster than 3. Similar studies using phosphate buffer were performed, displaying comparable water addition results. Measurements of the time-dependent intracellular accumulation and DNA platination in the A2780 and the A2780R cells have shown that compound 2 is taken up in larger amounts by these cells, forming also larger amounts of DNA adducts than compound 3 does. Surprisingly, compound 3 is able to partially overcome the cisplatin resistance present in the A2780R, while 2 displays cross-resistance to cisplatin under the same conditions.

* Parts of this chapter are based on:
4.1. Introduction

From past research it is known that changing the chloride anionic ligands of cisplatin for carboxylato-type leaving groups, results in an improved water solubility, as well as in a lower toxicity, as shown for carboplatin.\textsuperscript{1-3} This approach is also explored for \textit{cis}-[Pt(A9pyp)(dmso)Cl\textsubscript{2}] (2), where the two chloride ligands have been substituted for a chelating cyclobutanedicarboxylate leaving group, forming [Pt(A9pyp)(dmso)(cbdca)] (3). In this formula A9pyp stands for E-1-(9-anthryl)-3-(2-pyridyl)-2-propenone.

Carbonate is present in relatively high concentration in the cellular medium as HCO\textsubscript{3}\textsuperscript{−}, in the cells and in the extracellular fluids. Moreover, recently it has been reported to be involved in the hydrolysis products of cisplatin and carboplatin.\textsuperscript{4} In addition, the influence of carbonate on the binding of carboplatin and cisplatin has been reported,\textsuperscript{5-7} where the carbonate is reported to be responsible for different reactivities of these drugs towards DNA. Therefore, a carbonate species might modulate the cytotoxic activity of these drugs. Similar studies have been performed using phosphate buffer, which was also found to influence the DNA interaction.\textsuperscript{7}

Different solution studies using NMR spectroscopy have been performed to study the reactivity of compounds 2 and 3 in the presence of carbonate and phosphate species. The hydrolysis reaction of these compounds is important for the biological activity, since their hydrolysis may influence their cellular uptake, which may in turn influence the amount of compound reaching the biological target.

Several \textit{in vitro} studies of compound 3 have been performed in a pair of human carcinoma cells, i.e. sensitive (A2780) and cisplatin-resistant (A2780R) cells, and compared to the dichloride analogue \textit{cis}-[Pt(A9pyp)(dmso)Cl\textsubscript{2}] (2). The ability of 2 and 3 to exhibit high cytotoxic activity against human ovarian carcinoma cell lines under different exposure conditions has been investigated in relation to their accumulation within these cells, and their interaction with cellular DNA. Significant differences have been found in the \textit{in vitro} studies of these two platinum(II) compounds, suggesting that the different leaving groups in 2 and 3 play an important role in their cellular processing.
4.2. Material and methods

4.2.1. Studies of hydrolysis by NMR spectroscopy

All NMR spectra were recorded using a Bruker DPX300 or DPX600 spectrometer using a 5-mm multi-nucleus probe. The temperature was kept constant at 20 ºC or 37 ºC by a variable-temperature unit. $^{195}$Pt chemical shifts were referenced to Na$_2$[PtCl$_6$] ($\delta = 0$ ppm).

Due to the poor solubility of both compounds in aqueous media, DMF-d$_7$ solutions containing cis-[Pt(A9pyp)(dmso)Cl$_2$] (2) or [Pt(A9pyp)(dmso)(cbdca)] (3) were prepared *in situ* and mixed with a carbonate buffer. Carbonate buffer was prepared dissolving NaHCO$_3$ (1.7 mg, 0.02 mmol) in D$_2$O (pH = 8.5). In a NMR tube the solutions of the compounds were mixed with carbonate buffer, so that the final concentration of carbonate was 25 mM, and the Pt:carbonate ratio was 1:3. Spectroscopic changes were followed over time using $^1$H NMR spectroscopy at 37 ºC. In addition, 2D NMR and $^{195}$Pt NMR spectroscopy were performed in order to investigate the reaction products from both compounds 2 and 3 and carbonate.

Phosphate buffer in D$_2$O (pH = 7.8) was also used to investigate its reaction with compounds 2 and 3 in solution. Phosphate buffer was prepared by dissolving Na$_2$HPO$_4$ (7.06 mg, 0.05 mmol) and KH$_2$PO$_4$ (6.78 mg, 0.05 mmol) in D$_2$O as previously described. $^1$H NMR, 2D NMR and $^{195}$Pt NMR spectroscopy were also performed to study the reactions of both compounds 2 and 3 with phosphate, under the same conditions as described for hydrogencarbonate experiments.

4.2.2. Platinum complexes

The compound K$_2$PtCl$_4$ was used as obtained from a loan scheme with Johnson and Matthey (Reading, UK). The platinum(II) compounds cis-[Pt(A9pyp)(dmso)Cl$_2$] (2) and [Pt(A9pyp)(dmso)(cbdca)] (3) were prepared as described in chapter 2. 20 mM stock solutions of 2 and 3 in DMF were freshly prepared before use in their *in vitro* studies. In all experiments, the compounds were added to the cell culture medium, so that the final DMF concentration did not exceed 0.5% (v/v), which was found to be not cytotoxic for the cells. Cisplatin (cis-[PtCl$_2$(NH$_3$)$_2$]) was purchased from Aldrich and served as a
reference compound. Aliquots of a 2 mM stock solution of cisplatin in 40 mM NaCl were stored in the dark at -20 °C, defrosted and diluted with complete medium to the desired final concentration before use.

4.2.3. Cell lines, culture conditions and cytotoxicity assay

The human ovarian carcinoma cell line A2780 and its cisplatin-resistant counterpart A2780R were grown as monolayers at 37 °C in a 7% CO₂ atmosphere, and were maintained in a continuous logarithmic culture as described in Chapter 3 (section 3.2.1). The cytotoxicity assay was performed as described in Chapter 3 (section 3.2.3).

4.2.4. Determination of cellular platinum accumulation

Cellular accumulation was determined in both cell lines exposed to cisplatin (cis-[PtCl₂(NH₃)₂]), cis-[Pt(A9pyp)(dmso)Cl₂] (2) and [Pt(A9pyp)(dmso)(cbdca)] (3) at the final concentration of 50 μM in complete medium. The assay has been described in Chapter 3 (section 3.2.4). The results were related to the used number of cells (nanogram platinum per million cells).

4.2.5. Measurement of DNA platination

Ten million cells were incubated with cisplatin, cis-[Pt(A9pyp)(dmso)Cl₂] (2) and [Pt(A9pyp)(dmso)(cbdca)] (3) at the final concentration of 50 μM in complete medium up to 1 h. The assay has been described in Chapter 3 (section 3.2.5). The DNA concentrations were determined by measuring the UV absorption at 260 nm, and the platinum content in the samples was measured by FAAS.

4.2.6. Binding to calf thymus DNA

For each rₜ (rₜ is defined as the number of platinum bound per nucleotide residue) determination, 100 μg of calf thymus DNA in miliQ water was treated with 80 μM of cisplatin, cis-[Pt(A9pyp)(dmso)Cl₂] (2) and [Pt(A9pyp)(dmso)(cbdca)] (3), in a total volume of 100 μl. The assay has been described in Chapter 3 (section 3.2.6).
4.2.7. Statistical analysis

In the biological studies such as platinum accumulation, DNA platination, and cytotoxic activity, the Student’s t-test was performed for statistical comparison. A \( p \)-value < 0.05 was considered as statistically significant.

4.3. Results and discussion

4.3.1. NMR studies of hydrolysis in the presence of NaHCO₃

(i) \(^1\)H NMR spectroscopy:

As in the case of carboplatin, the \([\text{Pt}(\text{A9pyp})(\text{dms})(\text{cbdca})]\) (3) has a dicarboxylate chelate ligand as leaving group, making it supposedly less reactive in aqueous media than 2. It has been reported that the hydrolysis rate of carboplatin follows a pseudo-first-order rate constant in water at 37 °C, being much slower than the hydrolysis rate of cisplatin.\(^4\)\(^9\)-\(^1\)\(^1\) The results of several studies lead to the conclusion that for activation of carboplatin it is required to obtain ring-opened compounds that subsequently can interact with the biological target.\(^1\)\(^1\) More recently, it has been suggested that the different carbonate forms in the blood (CO\(_3^{2−}\); in equilibrium with hydrogen carbonate, HCO\(_3^{−}\); carbonic acid, H\(_2\)CO\(_3\); and dissolved carbon dioxide, CO\(_2\)) might be responsible for the activation of carboplatin.\(^4\)\(^7\) To investigate the role of the carbonate species in the reactivity of cis-[Pt(A9pyp)(dms)(Cl\(_2\)]) (2) and [Pt(A9pyp)(dms)(cbdca)] (3), several NMR spectroscopic studies have been performed using NaHCO\(_3\) dissolved in D\(_2\)O (pH = 8.5), which are described in detail below.

The ligand A9pyp is a completely conjugated system (Fig. 4.1), and the protons corresponding to the carbon-carbon double bond (H(8) and H(9)) give two doublets in the aromatic region on the \(^1\)H NMR spectrum. The addition of a carbonate solution to the DMF-d\(_7\) solution of cis-[Pt(A9pyp)(dms)(Cl\(_2\)]) (2) or [Pt(A9pyp)(dms)(cbdca)] (3) surprisingly results in the loss of these two doublets, as clearly shown in the spectra displayed in Fig. 4.2. Spectroscopic evidences suggest that the C(8)=C(9) bond undergoes a water addition when 2 and 3 are mixed with carbonate buffer. These evidences are discussed in detail below.
Fig. 4.1. Schematic representation of the water addition across the carbon-carbon double bond C(8)=C(9) in the presence of carbonate buffer (L stands for ligand).

Fig. 4.2. Spectroscopic changes of \textit{cis}-[Pt(A9ppy)(dmso)Cl$_2$] (2) (top spectra) and [Pt(A9ppy)(dmso)(cbdca)] (3) (bottom spectra) in the aromatic region of the $^1$H NMR (300 MHz) 24 h after addition of carbonate buffer (spectra b) to the DMF-d$_7$ solution (spectra (a)). The reaction of 3 is slower, displaying new peaks (denoted with *) in addition to the peaks of possible intermediate species.
Together with the changes in the aromatic region in the $^1$H NMR spectrum of both compounds, three new peaks at 6.04 ppm, 3.56 ppm and at 3.49 ppm in the $^1$H NMR spectrum of [Pt(A9ppy)(dmso)(cbdca)] (3) in DMF-$_2$-carbonate buffer appear in the course of the reaction (Fig. 4.3). In the case of cis-[Pt(A9ppy)(dmso)Cl$_2$] (2), likewise new peaks at 6.02 and 3.67 ppm and 3.62 ppm appear, almost immediately after the addition of carbonate (Fig. 4.4, spectrum a), with no further changes over time (Fig. 4.4, spectra b and c). Thus, compound 2 reacts significantly faster than compound 3 in the presence of carbonate.

**Fig. 4.3.** Time-dependent $^1$H NMR spectra (300 MHz) of [Pt(A9ppy)(dmso)(cbdca)] (3) upon the addition of carbonate (ratio 1:3). Spectrum (a) 15 min after the carbonate addition, (b) 2.5 h after addition and (c) 10 h after the addition. The symbol * shows the formation of the new species over time.

**Fig. 4.4.** Time-dependent $^1$H NMR spectra (300 MHz) of cis-[Pt(A9ppy)(dmso)Cl$_2$] (2) upon the addition of carbonate (ratio 1:3). Spectrum (a) 15 min after the carbonate addition, (b) 2.5 h after addition and (c) 10 h after the addition.
(ii) 2D NMR spectroscopy:

To determine the nature of the new peaks observed after the carbonate addition to compounds 2 and 3, the $^{13}$C {$^1$H} NMR spectra of the reaction mixture (24 h after mixed) of both compounds were recorded.

In the case of compound 3, the $^{13}$C {$^1$H} NMR spectra (Fig. 4.5) shows that the peak at 6.04 ppm belongs to a CH group, while the peak at 3.56 ppm corresponds to a CH$_2$ group (Fig. 4.5). Moreover, the peak at 3.56 ppm is also assigned to a CH$_3$ group from the dmso ligand, as well as is the peak at 3.49 ppm. Therefore, the new peaks appearing in the NMR spectra are clearly assigned to the formation of a new product, in which the ligand looses the conjugation (Fig. 4.1), explaining the disappearance of the doublets from H(8) and H(9) in the aromatic region.

![Fig. 4.5. 2D $^{13}$C {$^1$H} NMR spectrum (300 MHz) of [Pt(A9ppy)(dmso)(cbdca)] (3) 24 h after the carbonate addition. Cross peaks between the H(8) proton and the C(8), and the H(9B) and the C(9) from the water addition across the C(8)-C(9) bond are indicated (line). The CH$_3$ groups from the dmso ligand are separately indicated (encircled).](image)

Additional information for this unusual water addition reaction came from the $^{13}$C NMR spectrum (spectrum not shown) of 3 in DMF-d$_7$. The chemical shift of C(8) and C(9) of the double bond are initially observed at 143 ppm and 135 ppm, respectively. After the addition of carbonate buffer to the DMF-d$_7$ solution of compound 3 the $^{13}$C
spectrum (spectrum not shown) show a major change in the C(8) and C(9) chemical shifts to 80 ppm and 56 ppm, respectively.

In the case of the reaction between 2 and hydrogencarbonate, the new peaks at 6.02 ppm and 3.62 ppm are also assigned to the water addition across the carbon-carbon double bond. In fact, the peak at 3.62 ppm coincides also with one of the methyl groups of the dmso ligand, the second peak at 3.67 ppm also belongs to dmso, as confirmed using $^{13}$C{\textsuperscript{1}H} NMR spectroscopy (Fig. 4.6). Unfortunately, in the case of compound 2, the formation of a CH\textsubscript{2} group after the addition of water across the C(9) is less clearly visible in the $^{13}$C NMR spectrum. However, a cross peak in the $^{13}$C{\textsuperscript{1}H} spectrum is observed (i.e. the cross peak inside the square box of Fig. 4.6).

![Fig. 4.6. 2D $^{13}$C{\textsuperscript{1}H} NMR spectrum (300 MHz) of cis-[Pt(A9pyp)(dmso)Cl\textsubscript{2}] (2) 24 h after carbonate addition. Cross peak between the H(8) and the C(8) from the water addition across the C(8)=C(9) is indicated by the dotted line. The square show the cross peak between H9A/B and CH\textsubscript{2}, however, the CH\textsubscript{2} signal is not clearly seen in the $^{13}$C spectra-projection. Finally, the CH\textsubscript{3} groups from the dmso ligand are also indicated.](image)

Additional spectroscopic studies of the reaction of these new compounds with carbonate buffer were performed. 2D NOESY NMR spectroscopy studies of the reaction of compound 3 with carbonate reveal a cross peak between the new peak at 6.04 ppm
(CH group) and the peak at 3.56 ppm (Fig. 4.7). An additional cross peak between the peak at 6.04 ppm and H(6) from the pyridine ring is observed. These cross peaks indicate that the C(8) from the original carbon-carbon double bond has undergone an addition of an OH group. However, the peak at 3.56 ppm appears to correspond also to a methyl group of the dmsoligand. Interestingly, cross peaks between the peak at 3.56 ppm, the H(3) (proton in *ortho* to the nitrogen of the pyridine ring) and the peak of anthryl protons, suggest that the CH$_3$ protons of the coordinated dmsoligand are quite close to the pyridine ring and the anthryl ring.

![2D NOESY spectrum](image)

**Fig. 4.7.** 2D NOESY spectrum (600 MHz) of [Pt(A9pyp)(dmsoligand)(cbdca)] (3) 24 h after the addition of carbonate. The cross peak between the CH and the CH$_2$ from the water addition across the C(8)=C(9) bond is indicated with a solid line. Aromatic region 2D NOESY spectrum where a cross peak between CH and H(6) of the pyridine ring is indicated with a solid line. Cross peak between CH$_3$ of the dmsoligand with the H(3) and anthryl protons is indicated with a solid line.

The 2D NOESY spectrum of compound 2 shows again that the water addition across the C(8)=C(9) bond is comparable to that observed for compound 3. A cross peak
between the CH group and the CH$_2$ peak at 3.67 ppm is observed. Cross peaks between the new peak at 6.02 ppm (assigned to the CH) and the H(6) of the pyridine ring of the A9pypp ligand is observed (Fig. 4.8) as found for compound 3. Again, as in the case of compound 3, the peak at 3.67 ppm coincides with the methyl group of the dmso ligand, showing also in the 2D NOESY spectrum the cross peak between the H(3) (proton in ortho to the nitrogen of the pyridine ring) and this CH$_3$ group. In contrast to compound 3, the 2D NOESY spectrum of compound 2 after reaction with carbonate buffer shows a cross peak between the new peak of the CH$_2$ group and the anthryl protons.

![Fig. 4.8. 2D NOESY spectrum (300 MHz) of cis-[Pt(A9pypp)(dmso)Cl$_2$] (2) 24 h after the addition of carbonate.](image)

The cross peak between the CH and the CH$_2$ resulting from the water addition across the C(8)=C(9) bond is indicated by a solid line. The aromatic region 2D NOESY spectrum where cross peak between CH and H(6) of the pyridine ring is indicated by a solid line. The cross peak between CH$_3$ of the dmso ligand with the H(3), and the cross peak between CH$_2$ group and the anthryl protons, are indicated with a solid line.
The 2D NOESY spectra of compounds 2 and 3 suggest that both compounds form a similar reaction product; however, different conformations are observed, which might be expected given the different leaving groups.

It is important to mention that a control reaction was performed in which carbonate was added to the A9pyp ligand in absence of platinum(II) ion. In this case no changes were observed in the C(8)=C(9) bond up to 24 h after the addition of excess carbonate. Therefore, the platinum(II) coordination to the ligand must play an important role in the water addition to the C(8)-C(9) bond upon this carbonate addition. Interestingly, the water addition across the C(8)=C(9) bond is not observed by 1H NMR spectroscopy when 40% water in the absence of carbonate is added to the DMF solution to compounds 2 and 3 in absence of carbonate. Thus, it can be concluded that the modification of the alkene bond occurs also only when carbonate is present in solution.

(iii) 195Pt NMR spectroscopy:

To investigate the effect of the leaving groups on the carbonate addition in compounds 2 and 3, time-dependent studies using 195Pt NMR spectroscopy after the addition of carbonate buffer to the DMF-d7 solution containing the platinum compounds were performed. The 195Pt NMR spectra were recorded every 2.5 h, during the first 10 h of reaction with carbonate buffer.

As described in Chapter 2, the 195Pt NMR spectrum of compound 3 displays a single peak at -2490 ppm when dissolved in DMF-d7. When carbonate buffer is added to a solution of 3, a new peak at -2422 ppm (Fig. 4.9) is gradually appearing with a peak remaining at -2488 ppm corresponding to the unreacted 3. The difference in the platinum chemical shift of these two species is small, suggesting that the peak at -2422 ppm may also correspond to a donor set [NO2S] around the platinum.12 It is noteworthy that the hydrolysis of [Pt(A9pyp)(dmso)(cbdca)] (3) in DMF-d7 solution with 40% water in the absence of carbonate was also followed over time by 195Pt NMR spectroscopy. Only the peak of the authentic sample in DMF was observed even after 20 h, indicating the stability towards hydrolysis of [Pt(A9pyp)(dmso)(cbdca)] in 40% water. In contrast, changes are observed in the 195Pt NMR spectrum when carbonate is present in the DMF solution of 3, displaying a new peak at -2422 ppm.
Therefore, compound 3 is reacting in the presence of carbonate species in solution, suggesting that the cbdc ligand might be displaced from the coordination sphere of the platinum. This hypothesis may be supported by the $^1$H NMR spectrum of compound 3 in the presence of 3 equivalents of carbonate in 600 MHz, where alterations in the cbdc chemical shifts (Fig. 4.10) are observed.

![Fig. 4.9. Time-dependent $^{195}$Pt NMR spectra (300 MHz) of [Pt(A9ppp)(dms)(cbdc)] (3) 2.5 h, 5 h, 7.5 h and 10 h after the addition of carbonate (ratio 1:3).](image)

Compound 3 in DMF-d$_7$ shows the cbdc ligand chemical shifts at 2.48 ppm, 1.83 ppm and 1.50 ppm (see chapter 2). After the addition of carbonate buffer, splitting of the peaks is observed in this region, clearly indicating modifications in the cbdc ligand environment.

![Fig. 4.10. $^1$H NMR spectrum (600 MHz) of the cbdc region of compound 3 24 h after the addition of carbonate buffer. All the peaks corresponding to cbdc ligand are indicated with the * symbol.](image)
In the case of \textit{cis}–\textit{[Pt(A9pyp)(dmsol)Cl2]} (2), the $^{195}$Pt NMR spectrum in DMF-d$_7$ displays a single peak in the $^{195}$Pt NMR spectrum at -2832 ppm. Changes after addition of 3 equivalents of carbonate are observed in the time-dependent $^{195}$Pt NMR spectra. These studies show a new peak at -2712 ppm appearing after 2.5 h, together with a small peak at -2435 ppm. In contrast to compound 3, the peak corresponding to the unreacted compound 2 is not observed anymore after 2.5 h. This observation clearly indicates a higher reactivity of compound 2 as compared to the studies with compound 3.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig_4.11.png}
\caption{Time-dependent $^{195}$Pt NMR spectra (300 MHz) of \textit{cis}–\textit{[Pt(A9pyp)(dmsol)Cl2]} (2) 2.5 h, 5 h, 7.5 h and 10 h after the addition of carbonate (ratio 1:3).}
\end{figure}

These new peaks suggest also that the chloride ligands in compound 2 dissociate from platinum(II) in the presence of carbonate. The hydrolysis of \textit{cis}–\textit{[Pt(A9pyp)(dmsol)Cl2]} (2) in DMF-d$_7$ where 40% (v/v) of water is use in the absence of carbonate was also followed by $^{195}$Pt NMR over time, and compared to the spectra in the presence of hydrogen carbonate. Only after 20 h a new peak at -2789 ppm is observed; however, the peak corresponding to the authentic sample in DMF-d$_7$ at -2832 ppm remains visible even after this time (spectra not shown). Therefore, it is clear that the reactivity of the chloride leaving groups is faster in the presence of carbonate species. To investigate the species formed when the chloride ligands are deliberately removed in the presence of water, 2 equivalents of AgClO$_4$ in 40% of water were added to the DMF solution of 2. After 24 h the precipitated AgCl was filtered out and the $^{195}$PtNMR spectrum of the clear solution was recorded giving a peak at -2408 ppm. This observation suggests that the hydrolysis of 2 is not complete in 40% water, and that only one chloride
ligand is replace by a water molecule, yielding the above mentioned peak at -2789 ppm.\textsuperscript{12} This peak might be compared to the one observed in the presence of carbonate at -2712 ppm. Moreover, the peak observed at -2435 ppm may correspond to a new species where both chloride ligands are substituted, yielding a [NSO\textsubscript{2}] environment.\textsuperscript{12}

\textbf{4.3.2. Studies of hydrolysis in the presence of phosphate}

Reactions of carboplatin with phosphate (100 mM, pH = 7.0) are known in the literature.\textsuperscript{11} In order to investigate the reactivity of the cbdca chelating ligand of compound 3 in the presence of phosphate, different NMR spectroscopic studies were performed, as described for carbonate studies.

The addition of phosphate in a final concentration of 35 mM in the NMR tube containing compound 3 (ratio Pt:phosphate 1:5) dissolved in DMF-d\textsubscript{7} generates the same species in solution as the addition of carbonate does, as deduced from the \textsuperscript{1}H NMR and \textsuperscript{195}Pt NMR spectroscopy (spectra not shown).

The solution behavior of compound 2 in the presence of 3 equivalents of phosphate was also investigated using NMR spectroscopy techniques. Again the same species is observed after the addition of phosphate to compound 2, as compared to carbonate addition, as confirmed using \textsuperscript{1}H NMR spectroscopy (spectra not shown). In addition, the \textsuperscript{195}Pt NMR spectrum of compound 2 after the addition of phosphate yields a single peak at -2719 ppm (Fig. 4.12). This peak is comparable also to the one found in the experiments of compound 2 with carbonate (Fig. 4.11). However, the small peak at -2435 ppm (see Fig. 4.11) observed after reaction with carbonate is not observed upon reaction with phosphate.

\textbf{Fig. 4.12.} \textsuperscript{195}Pt NMR spectrum (300 MHz) of \textit{cis}-[Pt(A9pyp)(dmso)Cl\textsubscript{2}] (2) 14 h after the addition of 3 equivalents of phosphate buffer (pH 7.8).
Therefore, both addition of carbonate and phosphate buffer to compounds 2 and 3 display a fast reactivity towards the carrier ligand A9pyp at the carbon-carbon double bond. However, compounds 2 and 3 show different reaction rates, since 2 reacts immediately, while 3 shows a slower reactivity with these buffers. Therefore, the cbdca chelating ligand affects the kinetics of formation of active species, which is relevant information for the understanding of the biological activity of these platinum(II) compounds.

4.3.3. Cytotoxic activity in short- and long-time exposure experiments

The cytotoxic activity studies of 2 and 3 were performed in parallel against human ovarian carcinoma sensitive and cisplatin-resistant cell lines (A2780 and A2780R). The EC₅₀ values after 1 h and 48 h incubation with the corresponding compounds are summarized in Table 4.1.

<table>
<thead>
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<th>compound</th>
<th>Long-time exposure</th>
<th>Short-time exposure</th>
</tr>
</thead>
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<td></td>
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<td>A2780R</td>
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<td>EC₅₀, mM</td>
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<tr>
<td>pEC₅₀</td>
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<td>2.49±0.05</td>
</tr>
<tr>
<td>EC₅₀, mM</td>
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<td>3.2 x 10⁻³</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td>1.69±0.04</td>
</tr>
<tr>
<td>EC₅₀, mM</td>
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<td>20.4 x 10⁻³</td>
</tr>
</tbody>
</table>

RF (resistance factor) = EC₅₀ (A2780R)/EC₅₀(A2780), nd = not determinable

As shown in Table 4.1, both platinum(II) compounds are highly active against both the A2780 and the A2780R cells. Statistical analysis of the long-time exposure cytotoxic activity of cisplatin compared to cis-[Pt(A9pyp)(dmso)Cl₂] (2) shows significant differences ($p < 0.01$) in the case of A2780 cell line, implying that cis-[Pt(A9pyp)(dmso)Cl₂] (2) has an even better antiproliferative activity than cisplatin with a lower EC₅₀ value (chapter 3, Table 3.1). In contrast, [Pt(A9pyp)(dmso)(cbdca)] (3)
shows no significant differences compared to cisplatin, which suggests that both compounds have similar cytotoxic activity against the A2780 cells. However, the opposite was observed when the cytotoxic activity against resistant A2780R cell line was studied, as significant differences \( (p < 0.0001) \) between cisplatin and \([\text{Pt}(\text{A9py})\text{(dmso)}\text{(cbdca)}] \) (3) were observed, indicating that the latter is a better cytotoxic agent in A2780R cells than cisplatin.

*Cis*-\([\text{Pt}(\text{A9py})\text{(dmso)Cl}_2] \) (2) shows partial cross-resistance to cisplatin in the A2780R cell line, since significant differences have been found between the pEC\(_{50}\) against the A2780 and the A2780R \( (p < 0.01) \). These data confirm the results presented in Table 3.1. On the contrary, compound 3 shows no significant differences between the pEC\(_{50}\) values against A2780 and A2780R, confirming that 3 overcomes the cisplatin-resistance present in A2780R cells. Furthermore, as the long-time exposure cytotoxic activity of 2 and 3 were directly compared, significant difference in their activity against A2780 \( (p < 0.001) \) and against A2780R \( (p < 0.01) \) cell lines were observed, it appears that *cis*-\([\text{Pt}(\text{A9py})\text{(dmso)Cl}_2] \) (2) is slightly more active than compound \([\text{Pt}(\text{A9py})\text{(dmso)(cbdca)}] \) (3).

Short-time exposure cytotoxicity test have been performed in parallel to the long-time exposure with the aim to investigate the relationship between the cytotoxic activity of these two platinum(II) compounds and their ability to be taken up rapidly by the cell. With this short-time exposure assay it is possible to observe whether the cells are able to recover from 1 h drug treatment during the post-incubation time (48 h) in the presence of drug-free medium. After 1 h exposure a decrease in the cytotoxic activity compared to long-time exposure EC\(_{50}\) values is observed for both compounds, suggesting that the cells are able to partly recover from the damage caused by these platinum(II) compounds. In addition, after short-time incubation \([\text{Pt}(\text{A9py})\text{(dmso)(cbdca)}] \) (3) shows partial cross-resistance with cisplatin, suggesting that the post-incubation with drug-free medium after a short treatment with the compound leads to partial cellular repair of the induced damage. This was not observed in the case of *cis*-\([\text{Pt}(\text{A9py})\text{(dmso)Cl}_2] \) (2), which again agrees with the data reported in Chapter 3.
4.3.4. Intracellular platinum accumulation in human ovarian carcinoma cells

The time-dependent cellular accumulation of compounds \(\text{cis-}[\text{Pt}(\text{A9pyp})(\text{dmso})\text{Cl}_2]\) (2) and \([\text{Pt}(\text{A9pyp})(\text{dmso})(\text{cbdca})]\) (3) in human ovarian carcinoma cells was studied and compared to that of cisplatin. As shown in Fig. 4.13, both compounds are highly accumulated in both cell lines compared to cisplatin.

![Graph showing intracellular platinum concentration](image)

**Fig. 4.13.** Intracellular platinum concentration in A2780 and A2780R cell lines after incubation for 15, 30 and 60 min with 50 \(\mu\text{M}\) final concentration of cisplatin (\(\text{cis-}[\text{PtCl}_2(\text{NH}_3)_2]\), \(\text{cis-}[\text{Pt}(\text{A9pyp})(\text{dmso})\text{Cl}_2]\) (2) and \([\text{Pt}(\text{A9pyp})(\text{dmso})(\text{cbdca})]\) (3) (mean ±SD, \(n = 2-4\)).

The cellular uptake plays an important role in the cytotoxic action, as it is the first biological barrier that platinum compounds encounter on the way to DNA or to other intracellular targets. Inhibition of drug uptake and/or increased drug efflux is one of the most significant resistant mechanisms in A2780R cells, as reported in the literature.\(^{13-15}\) Cisplatin has been used as a reference compound, finding that its accumulation is significantly lower in the resistant cell line after 15 min \((p < 0.001)\), after 30 min \((p < 0.01)\) and after 60 min \((p < 0.05)\) incubation, compared to the sensitive cell line, which is in agreement with earlier literature data.\(^{13-15}\) In the case of \(\text{cis-}[\text{Pt}(\text{A9pyp})(\text{dmso})\text{Cl}_2]\) a different behavior was observed. No significant differences in the accumulation of this new platinum(II) compound within sensitive and resistant cells were found (Fig. 4.13), which suggests that the partial cross-resistance observed in the long exposure cytotoxicity experiments (Table 4.1) might be due to resistance
mechanisms other than decreased accumulation. However, it might also be very well possible that efflux mechanisms are active during the long-time incubation experiments.

In the case of [Pt(A9pyp)(dms)(cbdca)] (3), the same behavior as for the chloride was observed, while no significant differences were found between the accumulation within the A2780 cells and the A2780R cells. [Pt(A9pyp)(dms)(cbdca)] (3) is able to circumvent the resistance present in A2780R cells after long-time incubation (Table 4.1), suggesting a different cellular processing than that of cis-[Pt(A9pyp)(dms)Cl2] (2). As reported in the literature, the intracellular uptake is largely determined by the carrier ligand and more so than the anionic leaving groups, and more interestingly, a correlation between the platinum drug accumulation and the lipophilicity of the carrier ligand has been reported, which could offer the potential to minimize the impact of the decreased accumulation resistance mechanism. In the case of 2 and 3 the same carrier ligand was used and similar coordination by the nitrogen of the pyridine ring takes place. Therefore, the differences in the intracellular accumulation between these two new platinum(II) compounds have to be related to the different leaving groups. The accumulation of 2 is significantly higher in both cell lines (A2780 and A2780R) compared to 3, which may be explained by slower labilization of the cbdca chelating ligand. Therefore, the uptake of [Pt(A9pyp)(dms)(cbdca)] (3) is enhanced compared to that found for cisplatin; however, the chloride leaving groups play an important role in the influx of the platinum compound within the cells, since higher amount of cis-[Pt(A9pyp)(dms)Cl2] (2) are accumulated for both cell lines.

4.3.5. DNA platination

The interaction of cisplatin and analogues with cellular DNA has been extensively studied (see review). It has been proposed that the cellular resistance to cisplatin and derivatives either has the capacity to hamper or limit the formation of platinum-DNA adducts, or the cells are able to repair or tolerate the lesions caused by the platinum agent once they are formed. To investigate the relationships between cytotoxicity and the ability of these new platinum(II) compounds to form platinum-DNA adducts, the DNA platination of cisplatin, cis-[Pt(A9pyp)(dms)Cl2] (2), and [Pt(A9pyp)(dms)(cbdca)] (3) in both the A2780 and the A2780R cells have been performed and the results are graphically presented in Fig. 4.14.
Fig. 4.14. Intracellular DNA platination of cisplatin (cis-[PtCl₂(NH₃)₂], cis-[Pt(A9pyp)(dmso)Cl₂] (2) and [Pt(A9pyp)(dmso)(cbdca)] (3) in A2780 and A2780R cell lines after 1 h incubation at 50 µM final concentration (mean ± SD, n = 2-4).

As expected from results of earlier DNA platination studies regarding cisplatin in several sensitive/resistant cell line pairs, the DNA platination by cisplatin in A2780R cells is significantly lower (p < 0.05) than that found in the sensitive counterpart A2780. This is also in agreement with the data presented in Chapter 3 for cisplatin, and with a lower degree of DNA platination by 2 in the A2780R compared to the DNA platination of 2 in the A2780 cells. As discussed above, cross-resistance to platinum has been observed in the A2780R cells upon exposure with cis-[Pt(A9pyp)(dmso)Cl₂] (2); therefore, it is possible that increased DNA repair mechanisms are partially responsible for the decreased activity in the A2780R cells.

The DNA platination found for [Pt(A9pyp)(dmso)(cbdca)] (3) shows no significant differences between the A2780 and the A2780R; i.e. the levels of platinum-DNA adducts are lower than those found for the chloride, albeit higher than those after cisplatin treatment. Moreover, the cytotoxic activity of [Pt(A9pyp)(dmso)(cbdca)] (3) shows that this compound overcomes cisplatin-resistance against A2780R cells upon long-time exposure (Table 4.1), suggesting that [Pt(A9pyp)(dmso)(cbdca)] (3) is not highly affected by DNA repair mechanisms. Given that cis-[Pt(A9pyp)(dmso)Cl₂] (2) and [Pt(A9pyp)(dmso)(cbdca)] (3) are supposed to form the same or similar DNA adducts, it is not clear why the chloride would be affected by DNA repair and the cbdca analogue
would not. Nevertheless, after short-time incubation and subsequent post-incubation with drug-free medium \([Pt(A9pyp)(dmso)(cbdca)]\) (3) shows cross-resistance to cisplatin, which is most likely a cellular recovery from the damage caused by this compound.

In addition, when the DNA platination degrees of 2 and 3 are compared, significant differences are observed in both cell lines. This observation is again in agreement with the cytotoxic activity summarized in Table 4.1, where after 1 h or 48 h incubation with the compounds, significant differences are found between both compounds in both cell lines, with \(cis-[Pt(A9pyp)(dmso)Cl_2]\) (2) being the more cytotoxic (lower EC\(_{50}\)). Studies regarding the kinetics of the interaction of carboplatin and cisplatin with DNA\(^{21}\) have shown that the chelating cbdca ligand becomes a more labile ligand as soon as the monoaquated form has been formed. In contrast to this, the two chloride ligands of cisplatin leave at similar rates, and an about 100-fold larger dose would be expected to be needed for carboplatin to form equivalent binding to the DNA. In vitro studies, on the other hand have shown that equal binding of cisplatin and carboplatin to DNA result in similar cytotoxic activities;\(^{21}\) however, a 20 to 40-fold higher dose of carboplatin was used to obtain such equivalent DNA binding. In particular both \(cis-[Pt(A9pyp)(dmso)Cl_2]\) (2), and \([Pt(A9pyp)(dmso)(cbdca)]\) (3) forms high levels of DNA platination. Nevertheless, under the same conditions, \(cis-[Pt(A9pyp)(dmso)Cl_2]\) (2) binds in a larger extent to DNA in both cell lines compared to its cbdca analogue.

### 4.3.6. Binding to calf thymus DNA

To investigate the effect of leaving groups in the two platinum(II) compounds upon interaction with DNA, calf thymus DNA samples were incubated for 1 h at 37 °C with a 80 \(\mu\)M final concentration of each compound. Subsequently, the DNA concentration was measured with UV and the platinum content was determined by ICP-OES. From this analysis, the \(r_b\) values were calculated and are depicted in Fig. 4.15 (\(r_b\) is defined as the number of molecules of platinum bound per nucleotide residue).

As shown in Chapter 3, compounds 1 (\(cis-Pt(A9opy)Cl_2\)) and 2 bind more efficiently to DNA than cisplatin, which is in agreement with the experimental data obtain with intracellular DNA platination (see Fig. 3.2 and 3.3).

Compound 3 shows less efficiency to bind to calf thymus DNA than cisplatin, under the same conditions. Significant differences were found between the \(r_b\) values of
compounds 2 and 3 ($p = 0.0004$), compound 2 being the most effective DNA-binder of these two new platinum(II) compounds. In addition, compound 2 binds more efficiently than compound 1 (chapter 3, Fig. 3.3).

![Graph showing drug molecules bound per base pair to calf thymus DNA after incubation with 80 μM for 1 h at 37 °C with cis-[Pt(A9pyp)(dmso)Cl2] (2) and [Pt(A9pyp)(dmso)(cbdca)] (3), as compared to cisplatin (mean ± SD, n = 3).]

In contrast, the platination efficacy of compound 3 is significantly lower than that of cisplatin ($p < 0.02$). This result must be related to the different hydrolysis rate of the leaving groups. Nevertheless, the intracellular DNA platination of compound 3 in both human ovarian carcinoma cell lines showed different results, with large amounts of DNA adducts formed by 3, compared to those of cisplatin. This observation may be related to the high intracellular accumulation of 3, since more of the compound is entering the cell, which would lead to more of it reaching the DNA.

4.4. Conclusions

Studies in solution of compounds 2 and 3 in the presence of carbonate or phosphate have been performed. A common reaction occurs for both compounds 2 and 3 with small excesses of both carbonate and phosphate, where the carrier ligand quite rapidly loses its conjugation by the addition of water at the exocyclic carbon-carbon double bond. This chemical modification has been identified using 1D and 2D 1H NMR spectroscopy. This very unusual reaction does not take place when the uncoordinated
ligand is dissolved in DMF-d$_7$ and treated with carbonate or phosphate, suggesting that the platinum(II) ion is required for the hydration of the double bond C(8)=C(9). Moreover, the addition of water only to the DMF solution of 2 and 3 shows no reaction of the C(8)=C(9) bond, confirming that either the carbonate or the phosphate are needed to modify this double bond.

Studies of the reactivity of both compounds using $^1$H and $^{195}$Pt NMR spectroscopy in the presence of carbonate or phosphate buffer show that compound 2 reacts immediately, while compound 3 displays slower reactivity. Interestingly, the same $^{195}$Pt NMR spectroscopic studies in the absence of carbonate or phosphate, adding water to the DMF solution of compound 2 show that 2 may undergo dissociation of only one chloride ligand, albeit much slower than in the presence of carbonate or phosphate. In contrast, compound 3 shows no market dissociation of the cbdca ligand upon addition of just water. Therefore, compounds 2 and 3 are highly affected only when carbonate or phosphate species are present.

Both compounds 2 and 3 are taken up quite efficiently by both cell lines although compound 3 accumulates in lower amounts compared to compound 2. This accumulation may be determined by the hydrolysis rate, which is faster for compound 2 than for compound 3. The DNA platination of compound 2 is also higher than that of compound 3, in agreement with the differences in accumulation. Reduced amounts of compound entering the cell leads to less compound reaching the nuclear DNA and therefore, to a lower cytotoxic activity results. Interestingly, the biological activity of compound 3 shows that this compound is able to partly overcome the resistance to platinum in the A2780R cells, contrary to compound 2. The reasons behind the cross-resistance displayed by compound 2 are not fully understood. Nevertheless, compound 3 shows no significant differences between the DNA platination in the A2780 and the A2780R cells. This observation suggests that compound 3 is not or is less affected by DNA-repair mechanisms. The opposite was observed for compound 2, which strongly suggests that the cross-resistance to platinum indeed stems from DNA-repair mechanisms.
References