New fluorescent platinum (II) complexes containing anthracene derivatives as a carrier ligand : synthesis, characterization and in vitro studies

Marqués Gallego, P.

8.

Summary, general conclusions and future perspectives

8.1. Design of new platinum-based drugs

Despite the success of cisplatin as an anticancer agent, two important drawbacks of its clinical use have stimulated the design of new platinum-based therapeutics. As a consequence an enormous number of new platinum-based compounds has been synthesized aiming at: (i) high biological activity against cancers in which cisplatin is less active, (ii) high activity against cell lines resistant against currently available platinum derivatives.

Using the available information regarding structure-activity relationship of the currently known platinum drugs and the better understanding of the resistance mechanisms (both discussed in the introduction of this thesis), a rational design of new platinum-based drugs has been followed.

8.2. Summary

Chapter 1 deals with an overview of the most relevant findings regarding the mode of action of the classical platinum drugs, namely cisplatin, carboplatin and oxaliplatin. Moreover, the clinical drawbacks of cisplatin are described, and the approaches undertaken over the last years towards the design of new platinum-based anticancer compounds are discussed.

In part I of this thesis the synthesis, characterization and *in vitro* studies of a family of new platinum(II) compounds containing 9-anthryl pyridyl enones as a carrier ligand are described.

In **Chapter 2** the synthesis and full characterization of these new platinum(II) compounds are described. The carrier ligands are E-2-[1-(9-anthryl)-3-oxo-3-prop-2-enylpyridine] (A9opy, **L1**) and its "reverse" enone E-1-(9-anthryl)-3-(2-pyridyl)-2-

propenone (A9pyp, **L2**), both containing an anthracene ring linked to a pyridine ring. This unit is used to bind to platinum(II) ion in all the compounds. The compounds *cis*-[Pt(A9opy)Cl_2] (**1**) and *cis*-[Pt(A9pyp)(dmso)Cl_2] (**2**) differ in their carrier ligand, while the compounds **2** and [Pt(A9pyp)(dmso)(cbdca)] (**3**) differ in their leaving groups.

The solvolysis studies of **1**, **2** and **3** in DMSO-d_6 using ^{1}H NMR, ^{195}Pt NMR and ESI-MS spectroscopy are described in Chapter 2. Compounds **1** and **2** show fast solvolysis in DMSO-d_6, with the release of the carrier ligand and formation of [Pt$(dmso)_2Cl_2$] as the final product. The difference in the solvolysis reaction for **1** and **2** is explained based on their chemical structure. In contrast, it is concluded that the spectrum of compound **3** in DMSO-d_6 shows no changes over time. Therefore, the cbdca leaving group stabilizes the coordination sphere of the platinum(II) ion, compared with the chloride leaving groups.

To conclude Chapter 2 the cytotoxic activity of **1**, **2** and **3** in a panel of human tumor cells is described. Moreover, both free carrier ligands (**L1** and **L2**) are included in the cytotoxic tests, and cisplatin is used as a reference compound. The activity of all the new platinum(II) compounds is high. However, the ligand **L2** shows also high cytotoxicity, suggesting that the activity of **2** and **3** may result from the presence of the carrier ligand.

Chapter 3 deals with the biological activity of compounds **1** and **2** in a human ovarian carcinoma cisplatin-sensitive (A2780) cell line and its cisplatin-resistant counterpart (A2780R). Significant differences have been observed between compounds **1** and **2** in the *in vitro* studies, where the chemical structure seems to play an important role. Both compounds are highly active compared to cisplatin in the A2780 and the A2780R cells. In addition, compound **1** overcomes platinum resistance in the A2780R cells, in contrast to compound **2**. Both compounds are taken up very efficiently by both cell lines. Nevertheless, significant differences have been found between compounds **1** and **2**. Whereas compound **1** is accumulated in larger amounts within the A2780R cells than within the A2780 cells, compound **2** shows similar accumulation in both the A2780 and the A2780R cells. Furthermore, both compounds **1** and **2** show higher levels of platinum-DNA adducts as compared to cisplatin, which is in agreement with their cytotoxic activity. Interestingly, compound **1** shows higher levels of DNA platination in the

A2780R cells, in which **1** is able to overcome platinum resistance. It is also clear that compound **2** forms less adducts with DNA within the A2780R cells, which is also in agreement with the results of cytotoxic studies, where **2** shows partial cross-resistance with cisplatin in the A2780R cells. To investigate the effect of the steric hindrance around the platinum(II) ion in the compounds **1** and **2** on their DNA binding, interaction with calf thymus DNA was undertaken. Compound **2** was found the most effective DNA binder of these two new platinum(II) compounds; this behavior was expected given that the structure of compound **2** shows a smaller steric hindrance around the platinum(II) ion, as compared to **1**. The data summarized in Chapter 3 suggest that compound **2** may be inactivated by different resistance mechanisms, such as DNA repair or active efflux mechanisms, while compound **1** may not be significantly affected by them.

Exchanging the two chloride leaving groups of *cis*-[Pt(A9pyp)(dmso)Cl_2] (**2**) by the cyclobutyldicarboxylate chelating ligand results in the compound [Pt(A9pyp)(dmso)(cbdca)] (**3**). This modification allows the study of the role of the leaving groups in the biological activity of the compounds containing A9pyp (**L2**) as a carrier ligand. **Chapter 4** describes several *in vitro* studies in the human ovarian carcinoma cell lines (A2780 and A2780R) performed with compounds **2**, **3** and with cisplatin. Compounds with two chloride leaving groups have been reported to hydrolyze faster than those with chelating carboxylate-type leaving groups (i.e. carboplatin).[1] In addition, it has recently been suggested that the presence of carbonate ions in the blood contribute to the "activation" of carboplatin, with the ring-opening of the cbdca chelating ligand.[2, 3] Compound **2** was found to react faster in the presence of carbonate, as compared to compound **3**. Moreover, both compounds showed a quite unusual modification of the carrier ligand: water addition across the C=C bond was observed by ^{1}H NMR spectroscopy, losing the conjugation over the carrier ligand. This modification of the carrier ligand was also observed when phosphate buffer is added to both compounds **2** and **3**.

Cytotoxic activity, intracellular accumulation, and DNA platination of compounds **2** and **3**, compared to cisplatin, are also described in Chapter 4. Time-dependent intracellular accumulation of these platinum(II) compounds showed that the leaving group affects cellular uptake, since **2** is accumulated in higher amounts within the A2780

and the A2780R cells, compared to compound **3**. In addition, the leaving groups also seem to influence DNA platination, given that compound **3** displays lower levels of DNA adduct formation as compared to compound **2**. Interaction of compounds **2** and **3** with calf thymus DNA showed that the presence of the cbdca ligand decreases the efficiency of this interaction, as **3** forms lower levels of DNA adducts than compound **2** and cisplatin. Therefore, it is not surprising that compound **3** displays a lower cytotoxic activity than its chloride analogue. Nevertheless, compound **3** shows a higher cytotoxicity in the A2780R cells compared to **2**, whereas **3** partly overcomes the platinum-resistance present in the A2780R cells. The results suggest again that compound **2** may be influenced by the DNA-repair mechanisms in the A2780R cells, displaying cross-resistance to with cisplatin.

In part II of this thesis the synthesis, characterization and *in vitro* studies of a new fluorescent platinum(II) compound (*cis*-[Pt(bapda)Cl_2]) are described. Besides the synthetic route and the spectroscopic characterization of this new compound, **Chapter 5** deals with the solvolysis studies of *cis*-[Pt(bapda)Cl_2] in DMSO-d_6. This compound is not stable in DMSO; one chloride ligand is replaced forming the $[Pt(bapda)(dmso)Cl]^+$ species over time, as confirmed by ^{195}Pt NMR and ESI-MS spectroscopy.

To conclude Chapter 5 the cytotoxic activity of *cis*-[Pt(bapda)Cl_2] and the ligand bapda in a panel of seven human tumor cells is described. Both *cis*-[Pt(bapda)Cl_2] and the ligand bapda show higher cytotoxicity than cisplatin under the same conditions in most of the cell lines.

In **Chapter 6** the study of the cytotoxic activity of *cis*-[Pt(bapda)Cl_2] in the human ovarian carcinoma cell lines, the A2780 and the A2780R is presented. This platinum(II) compound shows a high cytotoxic activity against both cell lines; however, cross-resistance to cisplatin in the A2780R was observed. To investigate whether glutathione (GSH) is able to inactivate *cis*-[Pt(bapda)Cl_2] within the A2780R cells, depletion of the GSH levels with L-BSO was performed. The results have shown no significant differences in the cytotoxic activity of *cis*-[Pt(bapda)Cl_2] in normal or GSH-depleted A2780R cells. Therefore, glutathione does not play an important role in the cross-resistance displayed by *cis*-[Pt(bapda)Cl_2]. In addition, interaction of

cis-[Pt(bapda)Cl_2] with 4 equivalents of GSH was followed by ^{195}Pt NMR over time, confirming that these two molecules do not interact.

Cellular processing of *cis*-[Pt(bapda)Cl_2] showed accumulation in lysosomes after a short incubation in the A2780R cells, and the free ligand bapda displays the same accumulation in the A2780R cells, suggesting that this sequestration is not a related to platinum. In contrast, the ligand bapda seems to be localized in the A2780 cell membrane for a longer time than its corresponding platinum(II) compound, which accumulates in lysosomes in the first hours. This observation suggests that the detoxification mechanisms of the A2780 cells respond differently to the platinum(II) compound.

Fluorescence microscopy studies of GSH-depleted A2780R cells show no differences compared to the A2780R cells. Nevertheless, different cellular processing of the ligand bapda in GSH-depleted A2780R cells was observed. Therefore, the concentration of GSH in the A2780R cells has an influence in the cellular processing of bapda, which is also reflected in its cytotoxic activity.

Interaction of *cis*-[Pt(bapda)Cl_2] with 9-ethylguanine (9-EtG) studied over time using ^{1}H NMR, ^{195}Pt NMR and ESI-MS spectroscopy has shown that such interaction is not favorable. The steric bulk of the ligand around the platinum(II) ion most likely hampers the approach of the 9-EtG molecule to the metal center and slows down the reaction, resulting in only small amounts of the [Pt(bapda)(9-EtG)Cl]Cl. Additionally, quenching of the *cis*-[Pt(bapda)Cl_2] fluorescence emission upon addition of calf thymus DNA was observed, suggesting that *cis*-[Pt(bapda)Cl_2] interacts with DNA via intercalation. The same results were obtained with the ligand bapda; therefore, it can be concluded that the interaction of this platinum(II) complex with DNA is driven mainly by the anthracene moieties. To conclude Chapter 6, intracellular DNA was isolated from the A2780 and the A2780R cells incubated with *cis*-[Pt(bapda)Cl_2]. The DNA platination of *cis*-[Pt(bapda)Cl_2] in both cell lines was compared to that of cisplatin under the same conditions. The DNA-adduct formation displayed by *cis*-[Pt(bapda)Cl_2] is higher in both cell lines, as compared to cisplatin. Surprisingly, higher levels of DNA platination in the A2780R cells than in the A2780 cells were observed for *cis*-[Pt(bapda)Cl_2], which appears to be not in agreement with the observed cross-resistance. Therefore, high

tolerance to the DNA damage might be involved in the cellular processing of this compound in the A2780R cells.

To complete part II of this thesis, intracellular accumulation of *cis*-[Pt(bapda)Cl_2] in the A2780 and the A2780R cells is described in **Chapter 7**. To investigate the role of the lysosomes in the intracellular transport of *cis*-[Pt(bapda)Cl_2], studies with bafilomycin A_1 and monensin have been performed. Bafilomycin A_1, an inhibitor of the vacuolar type H^+-ATPase has shown to affect the pH distribution in the A2780 and the A2780R cells, while monensin, an inhibitor of the Na^+ H^+ exchanger, does not affect the pH distribution in these cells (according to the acridine orange experiments). The intracellular accumulation of *cis*-[Pt(bapda)Cl_2] in bafilomycin A_1-treated cells shows no significant differences with that of bafilomycin A_1-free cells, suggesting that endocytosis is not likely to serve as a transport pathway for *cis*-[Pt(bapda)Cl_2] to enter in the A2780 or the A2780R cells. Nevertheless, clear changes in the distribution of *cis*-[Pt(bapda)Cl_2] in both cell lines has been observed using fluorescence microscopy in bafilomycin A_1-treated cells, as compared to bafilomycin A_1-free cells. This observation suggests that the pH might have an effect on the distribution of *cis*-[Pt(bapda)Cl_2] within the cell; however, this effect does not alter the amount of compound that accumulates within the cells. Monensin displays a significant effect on both the biological activity and the intracellular accumulation of *cis*-[Pt(bapda)Cl_2] in the cisplatin-resistant A2780R cells. The cellular processing of *cis*-[Pt(bapda)Cl_2] in the monensin-treated cells also appears to be different from that of monensin-free cells. These changes in the A2780 and in the A2780R monensin-treated cells are comparable to those observed when the cells are preincubated with bafilomycin A_1. The compound is accumulated in the acidic lysosomes; however, the compound is also localized in different parts of the cells. The altered intracellular drug distribution in resistant cells might partly result from the activity of an efflux pump that brings the levels of intracellular drug down to a concentration that falls within the capacity of the cytoplasmic organelles to sequester the drug.[4] This observation can explain the decrease drug uptake of *cis*-[Pt(bapda)Cl_2] observed in monensin-treated A2780R cells, where more compound is localized in the cytoplasm of the cells after monensin incubation. Larger amounts of drug are distributed all over the cells; therefore, the cells increase the efflux to balance the levels of compound between

cytoplasm and acidic compartments. Nevertheless, the cytotoxic activity of *cis*-[Pt(bapda)Cl_2] in monensin-treated A2780R cells is higher than in the monensin-untreated A2780R cells. This observation is not yet understood, since lower accumulation of *cis*-[Pt(bapda)Cl_2] in the monensin-treated A2780R cells should lead to lower cytotoxic activity. It is possible that larger amounts of *cis*-[Pt(bapda)Cl_2] reach its biological target in monensin-treated A2780R cells, since the monensin is able to alter the permeability of the internal membranes.

8.3. General conclusions, evaluation and future perspectives

The compounds described in part I of this thesis show that 9-anthryl pyridyl enones ligands are good alternatives as carrier ligands to increase the intracellular accumulation of the platinum compounds within the cells. Nevertheless, the cytotoxicity of compound **1** is achieved after 1 h of exposure and 48 h of post incubation with drug-free medium (Table 3.1), suggesting that this compound is highly toxic to the cells. Different modifications in this compound should be performed to decrease the toxicity of compound **1**, such as the introduction of less labile leaving groups, thereby decreasing its reactivity. Nevertheless, it is difficult to predict whether different modifications around the platinum(II) ion would improve the cytotoxic profile of compound **1**. As clearly shown in Chapter 4, the replacement of the chloride leaving groups of compound **2,** by the cbdca chelating ligand giving compound **3,** modify the biological activity of these type of compounds. The accumulation of compound **3** within the cells is significantly lower than that of compound **2**. In addition, the DNA platination of compound **3** is lower than that of compound **2**. Therefore, a lower activity of **3** would be expected against both the A2780 and the A2780R cell lines compared to compound **2**. Nevertheless, compound **3** is able to partly overcome platinum resistance in the A2780R, in contrast to compound **2**. Therefore, it is not clear why compound **2** displays cross-resistance to platinum in the A2780R. The design of new platinum-based antitumor drugs should not be based on random modifications; therefore, more studies to establish the mechanisms of action of compounds **1**, **2** and **3** are needed in order to synthesize derivatives with improved activity profiles.

The ligands A9opy (**L1**) and the A9pyp (**L2**) are easily synthesized. Both carrier ligands are fluorescent due to the anthracene moiety; consequently, compounds **1**, **2** and **3**

are also fluorescent. Cellular processing of these compounds, using fluorescent microscopy in the A2780 and the A2780R cells, has been attempted. Unfortunately, the fluorescent emission of these compounds is not intense, and it is difficult to discriminate between the auto-fluorescence of the cells and the compounds' emission. Moreover, these platinum(II) compounds are highly cytotoxic, which makes impossible to increase the concentration of the compounds in the culture medium to achive stronger fluorescence signals. Therefore, the biological activity of these compounds plays an important role towards the *in vitro* investigations. Unfortunately, fast and highly active compounds cannot be easily studied in living cells using optical techniques such as fluorescence microscopy.

In addition, compounds **1**, **2** and **3** are not water soluble, which is not desired when applied in biological systems. Modification of the leaving groups of these compounds could be performed in order to improve their water solubility. However, the replacement of the chloride leaving groups in compound **2** with a cyclobutyldicarboxylate chelating ligand was not found sufficient to obtain a water soluble analogue. Therefore, it is recommended to modify the carrier ligands by introducing groups that will increase their water solubility. Nevertheless, this modification may affect the intracellular accumulation of the corresponding platinum compounds. A decreased intracellular accumulation of the platinum compounds may lead to decreased DNA platination. Thus, lower levels of the platinum agent achieving the cellular target may lead to lower cytotoxic activity of these compounds.

In addition, A9opy (**L1**) was found to display moderate cytotoxic activity, in contrast to A9pyp (**L2**) that is highly cytotoxic for the A2780 and the A2780R cells. This observation suggests that **L2** is highly responsible for the biological activity of compounds **2** and **3**. On the other hand, the high cytotoxic activity of compound **1** in all the cell lines might be due to the coordination of **L1** to the platinum(II) ion.

Metal complexes containing propane-1,3-diamine derivatives as carrier ligand have been described in the literature.[5-7] The attachment of two anthracene rings to the propane-1,3-diamine moiety results in a fluorescent platinum(II) compound, which is highly active against several human cancer cell lines. Nevertheless, this compound displays cross-resistance to platinum in the A2780R cells. In order to understand the

cross-resistance to platinum in the A2780R cells, several *in vitro* studies are described in chapter 6 and 7. However, the results presented in this thesis are not sufficient to achive a complete understanding, and additional efflux studies or/and DNA repair studies should be performed.

The fluorescence emission of both the ligand bapda and *cis*-[Pt(bapda)Cl_2] is observed in the same wavelength range, making it more difficult to confirm that the cellular processing of *cis*-[Pt(bapda)Cl_2] is not the cellular processing of the ligand bapda. This is one of the major drawbacks of the fluorescent platinum compounds. A new type of platinum compounds, in which the fluorescence emission of the carrier ligand changes upon complexation with the platinum(II) ion, should therefore be investigated.

To conclude, several benefits from the structure of the carrier ligands A9opy (**L1**), A9pyp (**L2**) or bapda, namely their high lipophilicity and the anthracene intercalative moieties, may be partly responsible for the activity of the corresponding platinum compounds. Therefore, carrier ligands are helpful in the synthesis of new active platinum compounds. However, when the carrier ligand is by itself cytotoxic the question that generally arises is: why is the platinum needed? A rational answer to this question may be that the platinum(II) ion allows the formation of cross-links with the DNA, generating a strong distortion in the DNA helix. As generally believed, a platinum(II) complex like cisplatin is hydrolyzed after entering the cell, and the mono-and diaqua species are able to bind to different macromolecules, including DNA.[8] Structurally distorted DNA is more difficult to be repaired by the cell in a short time. As shown in Table 3.1 the ligand **L2** displays a lower cytotoxicity than its corresponding platinum(II) compound **2** after short-time incubation (1 h), while **L2** is as efficient as **2** after long-time incubation studies. Therefore, the binding to DNA provided by the platinum(II) ion is an effective process to generate cytotoxic activity.

References

1. Frey, U.; Ranford, J. D.; Sadler, P. J., *Inorg. Chem.* **1993,** 32, 1333-1340.
2. Di Pasqua, A. J.; Goodisman, J.; Kerwood, D. J.; Toms, B. B.; Dubowy, R. L.; Dabrowiak, J. C., *Chem. Res. Toxicol.* **2006,** 19, 139-149.
3. Todd, R. C.; Lovejoy, K. S.; Lippard, S. J., *J. Am. Chem. Soc.* **2007,** 129, 6370-+.

4. Come, M. G.; Bettaieb, A.; Skladanowski, A.; Larsen, A. K.; Laurent, G., *Int. J. Cancer* **1999,** 81, 580-587.
5. Akdi, K.; Vilaplana, R. A.; Kamah, S.; Gonzalez-Vilchez, F., *J. Inorg. Biochem.* **2005,** 99, 1360-1368.
6. Appleton, T. G.; Hall, J. R., *Inorg. Chem.* **1970,** 9, 1800-1806.
7. Appleton, T. G.; Hall, J. R., *Inorg. Chem.* **1972,** 11, 124.
8. Reedijk, J., *Proc. Natl. Acad. Sci. U. S. A.* **2003,** 100, 3611-3616.