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Metal Anticancer Compounds

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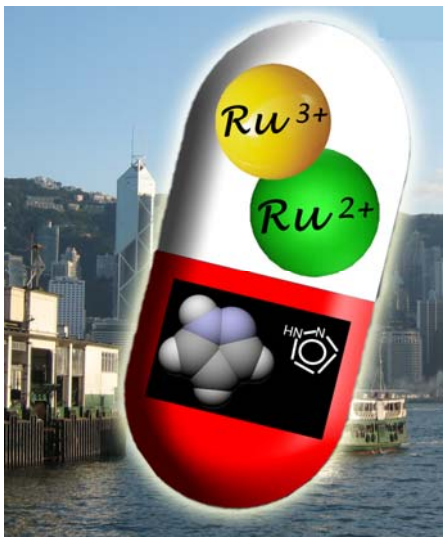

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The mechanism of action of platinum anticancer agents—what do we really know about it?

Dan Gibson

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Although 40 years have passed since the discovery of the anticancer activity of cisplatin, the mechanism of action of the drug is unclear. There are several working hypotheses that guide the researchers in this field. Unexpected results that we obtained cannot be reconciled with some of those assumptions. Our main intention is to call upon researchers in the field to re-examine the working hypotheses that may have been formulated on the basis of outdated methodologies.

Introduction

The accidental discovery by Rosenberg that platinum complexes can stop the proliferation of cells, led to the establishment of a new discipline in medicinal chemistry—metal based anticancer agents.[1,2] The intensive research in this field, that followed the discovery of cisplatin, has already yielded three FDA approved platinum anticancer drugs that are in widespread clinical use; cisplatin, carboplatin and oxaliplatin (Fig. 1).[3,4] Medicinal inorganic chemists have been working hard to develop new metal based drugs, and over the years more than 3000 platinum complexes were prepared as potential anticancer agents, and their biological properties were studied. Only about 30 compounds made it to clinical trials, some to advanced stages.[5]

Traditionally, heavy metals such as lead, mercury and cadmium were considered toxic, but Rosenberg's discovery has caused a major change in the thinking of inorganic chemists and prompted them to design and prepare heavy metal complexes as potential novel anticancer agents.

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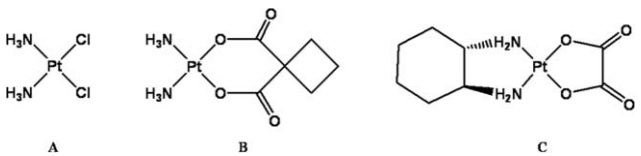

Fig. 1 The three clinically used Pt anticancer agents: (A) cisplatin, (B) carboplatin, and (C) oxaliplatin.

In parallel to the preparation and screening of platinum complexes as potential anticancer agents, extensive efforts were directed at elucidating the mechanism of action of platinum based drugs.[6,7] Only an understanding of the cellular events that take place as a result of exposure of cells to platinum drugs, might lead to new strategies for the preparation of novel platinum compounds with improved therapeutic profiles. Early investigations of the mechanism of action of cisplatin, suggested that the distortions in the DNA structure, caused by the covalent binding of cisplatin to two adjacent guanines on the same strand of the nuclear DNA, interfere with replication and trigger cellular events that lead to the death of the cancer cell.[8,9] Covalent modification of DNA by cisplatin was considered the most critical step in the chain of events that trigger cancer cell death and therefore, most of the research efforts over the years, were directed towards studying the interactions of cisplatin with DNA and elucidating the cellular responses to these interactions. With time it became evident that more efforts ought to be directed at studying non-DNA cellular interactions of platinum drugs and it was suggested that the mitochondria might be the critical pharmacological target of cisplatin.[10] Clearly, it is important to understand how the drugs enter the cells, to identify the different platinum species that are formed in the cytoplasm, to figure out how cisplatin manages to reach the nucleus despite the plethora of cytoplasmic platinophiles, to understand the consequences of forming non-DNA adducts in the cell and to elucidate the nature and the significance of the cellular responses to the distortion of the DNA.

Elucidating the mechanism of action of a cytotoxic compound is a very challenging task and even 40 years after the discovery of the anticancer activity of cisplatin, the picture is far from clear and some very basic questions remain unanswered. With the aid of recent advances in analytical, chemical, biophysical and biochemical technologies, more and more high resolution

information has been obtained, providing further insights into the mechanism of action of cisplatin.

The early paradigms and the structure activity relationships were formulated on the basis of research that was carried out in the 1970s and 1980s, that relied primarily on the chemical and biological methods that were available at the time, which unfortunately lacked the resolution and sensitivity necessary to portray an accurate picture of the cellular events. Although many of the details have yet to be unravelled there is a widespread agreement regarding the basic events that play important roles in the mechanism of action of cisplatin.[8] Some of these events, that take place after cisplatin enters the cell, are schematically depicted in Fig. 2. We now believe that cisplatin enters the cell by both passive diffusion and by the copper transporter hCtr1 pathway.[11] Once in the cytoplasm, which has a chloride concentration of only 4 mM, cisplatin undergoes aquation and hydrolysis reactions to generate various reactive platinum species. It is the aquated platinum complexes that are believed to bind to the nuclear DNA or to other weak nucleophiles such as carbonate and phosphates.[12] Cisplatin as well as the aquated complexes react readily with methionine, metallothioneins (MT) and glutathione (GSH).[13,14] Only a small fraction of the cellular cisplatin binds to the nuclear DNA and triggers cellular events that can lead to cell death.[7]

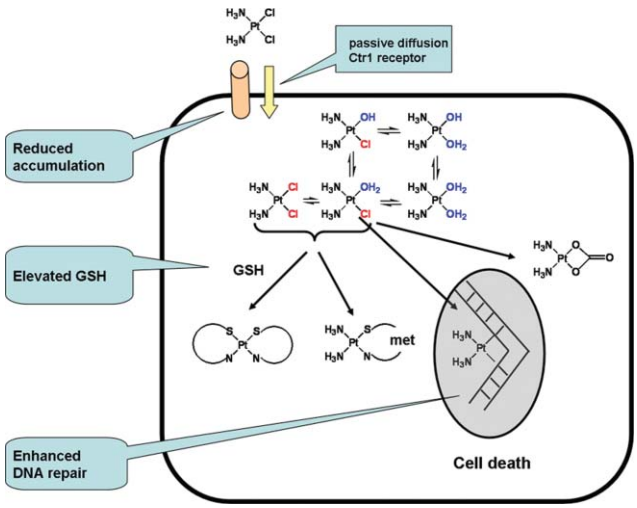

Fig. 2 A cartoon showing some of the cellular events that may occur when cells are exposed to cisplatin, beginning with entering the cell by passive diffusion or the Ctr1 receptor, through aquation and hydrolysis and binding to cellular nucleophiles. Also highlighted are the resistance mechanisms of reduced accumulation, cytoplasmic detoxification (mainly by GSH) and DNA repair.

One of the major problems encountered by clinicians when treating patients with platinum anticancer agents is that the tumors can acquire resistance to cisplatin, and do not respond to subsequent cycles of treatment by the platinum drugs.[15] In the case of cisplatin, three major resistance mechanisms were identified to date. They include reduced cell accumulation, cytosolic detoxification (mainly by GSH), and enhanced DNA repair and tolerance (Fig. 2).[16-19]

As scientists, we must first formulate working hypotheses that allow us to define the parameters necessary for designing new approaches towards unravelling the mechanism of action of the drugs. Many of the working hypotheses are based on the simplified scheme depicted in Fig. 2. In the course of research carried out in our laboratory, we obtained results which could not be reconciled with some of the basic assumptions regarding the cellular fate of cisplatin. This forced us to carefully examine the experimental data that led to these conclusions, and to ask ourselves "what do we really know about the mechanism of action of platinum anticancer agents?"

The main goal of this manuscript is to present the results that forced us to re-think some of the widely accepted notions and basic axioms regarding the mechanisms of action of platinum complexes and to encourage the readers to critically re-examine some of the "established paradigms".

How is the information of the cellular fate of platinum drugs obtained?

Many reviews describe the mechanism of action of cisplatin.[7-9] As more and more high resolution information regarding the mechanism of action of the drug is obtained, the picture that unfolds is becoming increasingly complex. We keep learning about the involvement of cisplatin in more biochemical processes and signalling pathways, but surprisingly little on the intracellular inorganic chemistry of cisplatin.

Most of the information on the mechanism of action of cisplatin comes from two major sources: (a) from biochemical methods and approaches applied to the study of biological fluids, cells or animals treated with cisplatin, or (b) from analyzing the reactions of cisplatin with biologically relevant nucleophiles in aqueous solutions, using conventional techniques such as UV, IR, X-ray crystallography and NMR spectroscopy for characterizing the products. The biochemical methods do not have the resolution necessary to uniquely characterize platinum adducts at the molecular level, and most of the high resolution chemical methods, lack the sensitivity and specificity necessary to characterize the platinum species in complex biological solutions.

Ideally, we would like to incubate proliferating cells with therapeutic concentrations of cisplatin and monitor non-invasively and in real time the reactions that occur inside the cells, and characterize the products that are formed. Cisplatin is a potent cytotoxic agent and often its IC_{50} values (the concentration of the drug in the growth medium that results in 50% cell death) are in the low μM range. Cisplatin not only reacts with nuclear DNA, its critical pharmacological target, but as an electrophile it can react with any of the available nucleophiles, and preferentially with sulfur containing ligands such as methionines and cysteines.[20] It seems safe to assume that cisplatin forms many different types of adducts in the cytoplasm. If the intracellular Pt concentration is 1 μM, and cisplatin forms only 10 types of chemically distinct adducts, the average concentration of each type of adduct might be around 100 nM, making it nearly impossible to characterize the platinum adducts that are formed in cells with the currently available experimental techniques (see below).

Since the platinum species that are formed in the cytoplasm cannot be directly characterized, how do we know for instance, the half life of cisplatin inside the cell, or whether cisplatin actually undergoes aquation and hydrolysis in the cytoplasm? We assume that the cytoplasm can be modeled by an aqueous solution, and that the fate of cisplatin in the cytoplasm can be predicted from studying the aqueous chemistry of cisplatin under "physiological conditions" (pH = 7 and 37 °C).

Our ability to critically evaluate reports describing "cellular events" involving platinum complexes depends on familiarity with the experimental techniques that are commonly used to characterize Pt complexes and on recognizing their strengths and weaknesses. Therefore, we will describe briefly some of the most important techniques used for characterizing Pt complexes and discuss their suitability for characterizing low levels of platinum complexes in biological fluids.

NMR spectroscopy

^{195}Pt NMR spectroscopy. Provides information on the oxidation state of the metal and on the nature of the atoms comprising the first coordination sphere of the metal.[21,22] Although it is an invaluable tool for characterizing Pt complexes in solution and an indispensable companion for the synthetic platinum chemist, ^{195}Pt NMR suffers from two major drawbacks that render it useless for monitoring the cytoplasmic interactions of cisplatin: (a) the low gyromagnetic ratio coupled with the 34% natural abundance of the ^{195}Pt isotope, make it rather insensitive with a limit of detection > 10 mM, (b) the efficient relaxation of square planar Pt(II) complexes by chemical shift anisotropy (CSA) makes it impossible to detect Pt that is bound to large nucleophiles.

[^{1}H,^{15}N] HSQC. Appleton and others have used the nitrogen chemical shifts of ^{15}N labeled cisplatin, *cis*-[$PtCl_2(^{15}NH_3)_2$], to obtain information on the types of atoms ligated to the Pt in a position that is *trans* to the labeled ammine.[23-25] ^{15}N NMR spectroscopy is very insensitive, even with 100% ^{15}N labeling, and is not really useful for studying low concentrations of Pt complexes. With the use of inverse detection 2D NMR spectroscopy, ^{15}N chemical shifts can be obtained with reasonable sensitivity. Sadler and Berners-Price have shown that [^{1}H,^{15}N] HSQC of *cis*-[$PtCl_2(^{15}NH_3)_2$], and other ^{15}N labeled Pt complexes, provide information on the type of atom that is bound *trans* to the labeled ammine.[26] It is easy to distinguish between oxygen, nitrogen or sulfur donors that are ligated to the Pt(II), *trans* to the labeled ammines. In addition, [^{1}H,^{15}N] HSQC is very useful for determining the oxidation state of the platinum. The proton chemical shifts of Pt(IV)-$^{15}NH_3$ complexes appear around 6–7 ppm while those of Pt(II)-$^{15}NH_3$ are around 3.5–4.5 ppm. Although this method is quite sensitive and allows observation of adducts in the 100 μM range,[27] it is still not sensitive enough to track Pt species inside cells. Other potential pitfalls in applying this method for the study of cytoplasmic interactions include possible *trans*-labilization of the labeled ammine ($^{15}NH_3$) due to binding to thiols or thioethers, resulting in the loss of the signal from the Pt complexes,[28-30] and the loss of the HSQC signal upon binding of the Pt complex to high molecular mass nucleophiles that tumble slowly, causing efficient relaxation, and broadening of the peaks. Although this technique is two orders of magnitude more sensitive than ^{195}Pt NMR spectroscopy, it does not provide as much information on the Pt coordination sphere.

Mass spectrometry. Mass spectrometry seems like the ideal method for characterizing the cytoplasmic adducts formed by cisplatin. MS is very sensitive and can easily detect sub femtomole quantities with resolutions that exceed 100,000. 2D-LC coupled with tandem mass spectrometry is one of the most powerful tools for protein identification and for the determination of post-translational modifications.[31] Mass spectrometry has been used to study the interactions of platinum complexes with proteins.[32-40] Although platination (of proteins) is in fact a post-translational modification, there are several potential problems in applying standard proteomic protocols to try and identify platinated proteins in cells or cell extracts. The standard protocols for enzymatic digestion of proteins by trypsin include the reduction of disulfide bridges with dithiothreitol (DTT) which might react with the platinum that is protein bound. The coordinative bond between the metal and the protein is weaker than the covalent peptide bonds, and might be broken during the reduction of the disulfide bridges by DTT, during the enzymatic digestions, during ionization (especially MALDI) or collision induced dissociation. Unfortunately, there are no analytical methods for isolating the platinated proteins, making it very difficult to identify the platinated proteins among the thousands of proteins in eukaryotic cells. Finding ways to separate the platinated proteins from the background of unplatinated proteins could significantly improve our ability to identify the cytoplasmic adducts by ESIMS.

Despite all the potential drawbacks mentioned, this approach has been successfully applied to identify 31 Pt-protein adducts resulting from incubation of cisplatin with *E. coli*.[41]

EXAFS and XANES. EXAFS provides information of the first coordination sphere around the Pt center. It cannot however clearly distinguish between nitrogen and oxygen donors or between chloride or sulfur ligands in biological fluids, and thus is somewhat less informative than [^{1}H,^{15}N] HSQC. EXAFS, however, is not limited by the size of the nucleophiles to which the platinum is bound and has been used to study metals bound to large proteins. The main problem with using EXAFS for monitoring platinum speciation in biological fluids is its inability to resolve mixtures of adducts with different coordination spheres, such as PtN_2Cl_2, PtN_2OCl, PtN_2S_2 PtN_4 *etc.* and the results will reflect the average of all the species.[42,43] Since cisplatin forms many types of adducts in the cell, EXAFS cannot be used to study Pt speciation in biological fluids. X-ray absorption near edge spectroscopy (XANES) can be used to identify the oxidation state of platinum complexes. Hambley showed that the XANES spectra of Pt(IV) and Pt(II) complexes have absorption profiles that differ primarily in the height of their edges, with the former displaying a much higher edge. They have successfully applied this technique for determining the Pt distribution and oxidation states in cells and spheroids.[44,45]

Attaching probes to platinum complexes. Various probes or reporter groups were attached to the platinum center in attempts to follow the intracellular fate of cisplatin. Usually fluorescent probes are tethered to the metal in order to study the intracellular Pt distribution using confocal microscopy.[46] Attaching a large probe such as a fluorophore or a photoaffinity tag, to cisplatin, which contains 11 atoms and not a single carbon atom, generates a new chemical entity that may have little in common with cisplatin. Hambley has shown that attaching an anthraquinone to platinum alters the distribution in spheroids.[47] Even if the probe does not alter the intracellular distribution of the drug, care must be taken to verify that the probe being monitored is still attached to the platinum.

Inductively coupled plasma–mass spectrometry (ICP-MS)

ICP-MS is a very sensitive method for the detection of atomic platinum. It can detect concentrations as low as 0.65 μg L^{-1}. It has been used extensively to quantify cellular uptake, DNA binding and binding to sub-cellular compartments.[48] It cannot however distinguish between the oxidation states of Pt nor can it provide any information of the coordination sphere of platinum and therefore it is of little use in the characterization of Pt complexes.

The half life of cisplatin in cells

It is has been shown that platinum anticancer agents enter cells by passive diffusion, or by the copper receptor hCtr1 or *via* cationic transporters.[11] Once inside the cell, cisplatin is believed to undergo a series of aquation and hydrolysis reactions resulting in several species that are in equilibrium (Fig. 2). Of particular interest is the mono-aquated species, *cis*-$[PtCl(H_2O)(NH_3)_2]^+$, that is believed to react with the nuclear DNA triggering apoptosis.[7-9] The equilibria between cisplatin and its aquation and hydrolysis products are known to exist in aqueous solutions, and were thoroughly studied.[49] But can the cytoplasm be considered just "an aqueous solution", and is it ever in a state of equilibrium? To the best of our knowledge, the various platinum species generated by aquation and hydrolysis of cisplatin in water, were never actually observed in cells. Considering the plethora of sulfur containing cellular nucleophiles, it is reasonable to assume that these platinophiles will react rapidly and irreversibly with cisplatin, and even more rapidly with the aquated species, preventing the establishment of any sort of equilibrium.

Cisplatin, *cis*-$[PtCl_2(NH_3)_2]$ does not react directly with DNA. It must first undergo a rate limiting aquation yielding *cis*-$[PtCl(H_2O)(NH_3)_2]^+$ that modifies the DNA.[49] Thus, it is important to know the half life for the formation of *cis*-$[PtCl(H_2O)(NH_3)_2]^+$ in the cytoplasm. The rate of aquation of cisplatin in water (or buffer) was measured, and the half life for formation of *cis*-$[PtCl(H_2O)(NH_3)_2]^+$ is around 120 min.[50] Sulfur containing nucleophiles in the cytoplasm are likely to react with cisplatin at a rate that is considerably faster than the rate of aquation, reducing the cytoplasmic concentration of *cis*-$[PtCl(H_2O)(NH_3)_2]^+$. Even today, 40 years after the discovery of the anticancer activity of cisplatin, we do not know such a basic thing as the half life of cisplatin in cancer cells. Unfortunately, this information cannot be easily obtained with the available experimental techniques.

In an attempt to gain some insights into this question, we used whole cell aqueous extracts as models for the cytoplasm, and measured the stability of cisplatin in the cell extracts. This was done by adding ^{15}N labeled cisplatin to the aqueous extracts of cancer cells and monitoring the disappearance of the peak of *cis*-$[PtCl_2(^{15}NH_3)_2]$ by $[^1H,^{15}N]$ HSQC NMR spectroscopy.[51] We measured the stability of cisplatin in the aqueous extracts of several different cell lines; A2780 (a human ovarian cancer cell line sensitive to cisplatin), A2780cisR (ovarian cancer line resistant to cisplatin) and HT-29 (human colon cancer cell line refractory to cisplatin) and HL-60. In all cases the $t_{1/2}$ for the disappearance of cisplatin was around 75 min (Fig. 3), which is significantly shorter than the half life reported for the first aquation of cisplatin in water. Interestingly, other than the peak of cisplatin we were unable to detect any peaks in the $[^1H,^{15}N]$ HSQC spectrum,

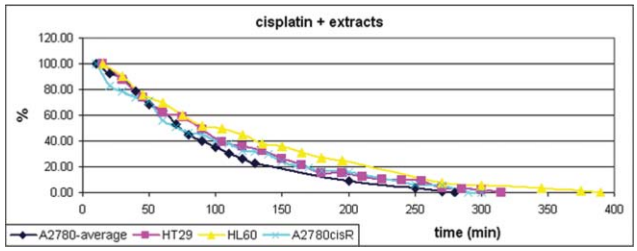

Fig. 3 The decay curves of the $[^1H,^{15}N]$ HSQC signal of ^{15}N labeled cisplatin incubated with cancer cell extracts at 37 °C, as measured by a 500 MHz NMR spectrometer. The half lives of cisplatin are approximately 75 min for four different cell lines. No peaks other than cisplatin were observed in the spectra.

and even with a high field instrument (900 MHz equipped with a cryoprobe), no peaks indicative of aquation were observed.[51] The inability to observe the peak of *cis*-$[PtCl(H_2O)(NH_3)_2]^+$ is not necessarily surprising and might be attributed to the low steady state concentration of *cis*-$[PtCl(H_2O)(NH_3)_2]^+$ resulting from its rapid reactions with cytoplasmic nucleophiles.

The role of glutathione in resistance to cisplatin

One of the major problems in the use of cisplatin as an anticancer agent is that tumors can acquire resistance to the drug. Often, cells resistant to cisplatin have elevated levels of cellular glutathione (GSH).[52] It therefore seems logical to conclude that GSH plays a major role in the mechanism of cellular resistance to cisplatin. The role that GSH might play in enhancing cellular resistance to cisplatin is still under debate. Early studies reported a correlation between the relative ineffectiveness of cisplatin in several ovarian cancer cell lines and elevated cellular levels of GSH.[50] Bednarski, concluded on the basis of studying 19 cancer cells lines, that there is no correlation between the potency of cisplatin and the levels of GSH in those cells.[54,55] A recent paper suggests that elevated GSH levels actually sensitize cells to cisplatin by up-regulating the copper transport hCtr1 receptor that facilitates cisplatin uptake into cells.[56]

HSAB (hard soft acid base) theory teaches us that cisplatin, being a soft metal has a high affinity to sulfur containing ligands.[57] Indeed, cisplatin reacts readily with GSH in aqueous solutions under "physiological conditions".[58-60] The intracellular GSH concentrations are in the mM range, orders of magnitude higher than the intracellular platinum levels that are in the low μM range, making it intuitively appealing to assume that GSH is the major cellular target of cisplatin, and that the reaction between them leads to the formation of stable covalent adducts that prevent the platinum from binding to DNA. This was the working hypothesis underlying the development of Picoplatin, *cis*-$[PtCl_2(NH_3)(2\text{-picoline})]$, an analog of cisplatin in which a bulky 2-methylpyridine replaces one of the ammine ligands thereby slowing down substitution reactions that proceed *via* a five coordinate trigonal bipyramidal intermediate.[61] Picoplatin, which is in clinical trials, was specifically designed to overcome cisplatin resistance by slowing its binding to cellular GSH. To the best of our knowledge there are no reports on its half life in cells, or on the nature of its major cellular targets and whether they differ from those of cisplatin.

The interactions of cisplatin with L1210 murine leukemia cells was studied by Ishikawa and Ali-Osman who concluded that after a 12 h incubation, 60% of the intracellular cisplatin reacted with GSH, that *trans*-labilized both ammine ligands to form the bis-(glutathionato)-platinum, [Pt(GS)$_2$] (Fig. 4).[62] Berners-Price and Kuchel studied the reactions of cisplatin with reduced GSH in red blood cells by NMR. They were unable to detect the formation of Pt-GSH adducts but saw a reduction in cellular GSH levels. This led them to conclude that Pt bound GSH was present in complexes with molecular masses higher than 10 kDa. They did not however, identify ternary protein-Pt-GSH complexes.[58]

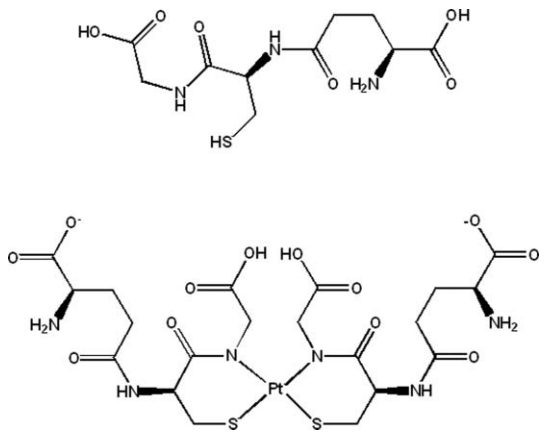

Fig. 4 Glutathione (top) and the proposed major cellular adducts formed by cisplatin, Pt(GS)$_2$ (bottom).

When we monitored the interactions of ^{15}N labeled cisplatin with whole cell extracts, we were unable to observe the formation of Pt-GSH adducts. However, when the labeled cisplatin was added to the low molecular mass fraction of the extracts (< 3 kDa), we detected the formation of Pt-GSH adducts.[51] The fact that the Pt-GSH adducts were observed in the low molecular mass fractions of the extracts but not in the whole extracts suggested that GSH may not be the major target of cisplatin in the cytoplasm. To further examine this possibility, we incubated whole cell extracts with cisplatin and separated the reaction mixture into low and high molecular mass fractions (either with a 3 kDa or 30 kDa filter) and quantified by ICPMS the Pt levels in each fraction. The results, depicted in Fig. 5, demonstrate that two thirds of the Pt adducts formed in the extracts have a molecular mass exceeding 3 kDa suggesting that it is unlikely that [Pt(GS)$_2$] accounts for 60% of all the platinum adducts. Moreover, at neutral pH [Pt(GS)$_2$] is anionic (Fig. 4) and should be present in the anionic fraction of the low molecular mass fraction which contains at most 20% of the Pt (Fig. 5). Also, if 60% of the cellular adducts were in the form of [Pt(SG)$_2$], we would expect to see 60% of the labeled ammine ligands labilized. In a separate study we quantified the *trans* labilization of ammines from cisplatin by cells and by cell extracts and were only able to observe 20% *trans*-labilization after 12 h.[29,30]

It should be noted that quantifying Pt-GSH adducts in cells or cell extracts is quite complex since cisplatin can first react with non-GSH cellular nucleophiles forming stable adducts that can proceed to react with GSH to form Pt mediated ternary complexes. Conversely, cisplatin may first react with one molecule of GSH, and this adduct may proceed to react with other cellular

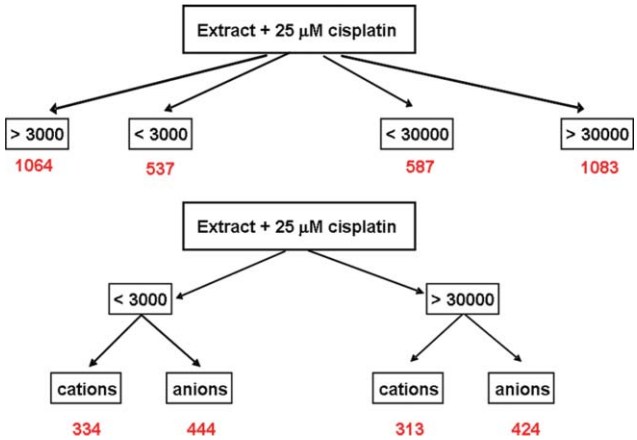

Fig. 5 A scheme of the fractionations that were carried out on the A2780cisR cell extracts that were incubated with cisplatin in order to quantify the Pt levels in each fraction. The red numbers under the boxes are the Pt levels in ng. Extracts were separated to low and high molecular mass by centricons (3 kDa and 30 kDa) and the Pt was quantified in each fraction (top). The Pt was also quantified in the cationic and anionic sub-fractions of the low and high molecular mass fractions of the extracts (bottom).

nucleophiles. We have shown that a platinated protein, ubiquitin, can react with GSH or GMP to form ternary complexes.[34,35] Therefore, we cannot rule out that the high molecular mass adducts may contain Pt-SG moieties. Our results differ from those reported by Ishikawa and Ali-Osman,[62] but it should be noted that the cell lines used were different and that the results of this study were obtained in cell extracts (in order to correlate them with the NMR studies), while the results reported by Ishikawa and Ali-Osman were obtained from intact cells.

Higher levels of GSH in resistant cell lines do not necessarily imply more efficient binding between cisplatin and GSH. Even in cancer cell lines that are sensitive to cisplatin, there is a huge excess (> 500 fold) of intracellular GSH compared with the intracellular concentration of cisplatin. So is the additional GSH that is present in resistant cell lines really necessary for inactivating cisplatin?

So, what role does GSH play in the mechanism of cisplatin resistance? In an attempt to explain why increased GSH levels, did not reduce the number of Pt-DNA adducts in the cells, it was hypothesized that GSH, through its function as an antioxidant, plays a role in apoptotic regulatory pathways.[63] In another study, reducing cellular GSH levels with BSO (inhibits GSH biosynthesis) did not affect the resistance of the cells, suggesting that although exposure of cells to cisplatin results in higher GSH levels, GSH is not directly involved in cisplatin resistance.[64] Contrary to the accepted notion that GSH is involved in resistance pathways, a very recent report suggests that high levels of GSH actually enhance the sensitivity of cancer cells to cisplatin by increasing the number of available hCtr1 receptors that enhance cellular accumulation of the drug.[56]

When we started our NMR work on cell extracts, our working hypothesis was that GSH is the major cellular target of cisplatin. This seemed both intuitive and consistent with the fact that cisplatin reacts rapidly with GSH to form stable covalent adducts and that GSH levels in cisplatin resistant cell lines are usually higher than those in sensitive lines. Much to our surprise we found that based on our work it seems that GSH may not be the major

target in cancer cell extracts and $[Pt(GS)_2]$ is not the major adduct of cisplatin in the cell extracts.

The nature of the cellular targets of cisplatin

It is likely that many different cellular nucleophiles react with cisplatin. In addition to the Pt-DNA adducts that are formed; there are low molecular mass nucleophiles containing sulfur ligands, such as GSH and metallothionein (6 kDa) that are presumed to be the major targets of cisplatin. Our results show that nearly two thirds of the platinum adducts formed from the incubation of cisplatin with cell extracts have molecular masses greater than 30 kDa. Interestingly, the half life of cisplatin in the high molecular mass fraction of the extracts is shorter than its half life in the low molecular mass fraction (75 *vs.* 90 min).[51] This is counterintuitive as it is expected that cisplatin will react faster with the smaller nucleophiles.

Since we cannot monitor platinum reactions inside cells, we have to work with some model of a cell. We have chosen, cell extracts as a model and we have shown that results obtained in cell extracts depend on the cell line used. It should be remembered that cell extracts might not be the ideal model for the cytoplasm as they lack the structure and organization that exist in a cell, meaning that Pt speciation inside cells may be different from that obtained in cell extracts.

Interesting information on the cellular targets of cisplatin came from a study where *E. coli* cells were treated with cisplatin, and multidimensional liquid chromatography and electrospray ionization tandem mass spectrometry were used to identify 31 proteins to which platinum was bound.[41] These included high abundance enzymes and ribosomal proteins, as well as DNA and RNA binding proteins. One of the most interesting results of that study was that contrary to what we expect from HSAB theory, that Cys and Met would be the primary binding sites for cisplatin on proteins, carboxylate and hydroxyl groups were identified as the platinum coordination sites in 18 out of 31 proteins and methionine was identified as the binding site only 9 times, and no binding to cysteine was reported. Cisplatin binding to these high molecular mass proteins is in agreement with the observations by Berners-Price and Kuchel,[58] and with our results. The mass spectral analysis revealed that the Pt fragments, which were found attached to the proteins, included only $[Pt]^{2+}$, $[Pt(NH_3)_2]^{2+}$ and $[Pt(NH_3)_2Cl]^{+}$ and that there was no evidence for a protein-Pt-GSH ternary complex.

Reduction of Pt(IV) prodrugs with axial acetato ligands

In is noteworthy, that the whole field of platinum anticancer drugs that is associated primarily with the three FDA approved Pt(II) complexes (cisplatin, carboplatin and oxaliplatin—Fig. 1), had its origins in a Pt(IV) complex, *cis*-$[PtCl_4(NH_3)_2]$.[1,2] It took over 20 years until significant systematic efforts were channeled towards preparation of Pt(IV) complexes as anticancer agents.[65] Octahedral platinum(IV) complexes, such as satraplatin and LA-12 (Fig. 6), are considered inert prodrugs that can be administered orally and are activated by reduction to their more reactive platinum(II) analogs.[66] Platinum(IV) complexes prefer a low-spin, d^6 octahedral geometry, that is substitution-inert, so reactions with biological nucleophiles are highly unlikely. Thus, administration of platinum

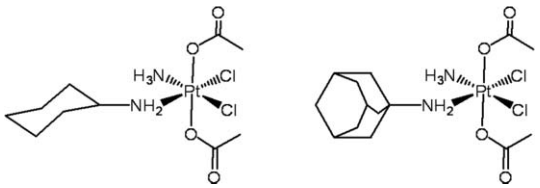

Fig. 6 Two of the most prominent Pt(IV) anticancer agents have the general formula *ctc*-$[PCl_2(OCOCH_3)_2(NH_3)(Am)]$ where Am = cyclohexylamine for satraplatin (left) and adamantylamine for LA-12 (right).

in the IV oxidation state is likely to increase its lifetime in the blood improving its chances of reaching the tumor intact.

The prevailing assumption is that inside the cell, the Pt(IV) prodrugs are reduced by low molecular antioxidants such as ascorbate and GSH to the more reactive Pt(II) analogs that end up modifying the nuclear DNA (Fig. 7).[66] Two parameters that are critical for the activation of the Pt(IV) are the reduction potential of the complex and the rate of reduction.

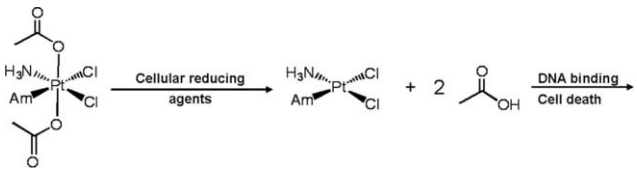

Fig. 7 The activation of the inert Pt(IV) prodrugs. In the cell the octahedral Pt(IV) complex undergoes a 2 electron reduction, presumably by ascorbate or GSH, to yield the semi-labile square planar Pt(II) derived from the parent Pt(IV) by the loss of the two axial acetato ligands. It is the diam(m)inedichloridoplatinum(II) complex that is responsible for the cytotoxic activity.

The reduction potential of complexes of the type *ctc*-$[PtCl_2L_2(NH_3)(Am)]$ (where L are the axial ligands) depends primarily on the nature of the axial ligand. The most easily reduced complexes are those with chlorido axial ligands, more difficult to reduce are complexes with axial acetato ligands and most difficult to reduce are complexes with hydroxido axial ligands.[67,68] Interestingly, two of the more promising Pt(IV) complexes, satraplatin and LA-12, have axial acetato ligands with intermediate reduction potentials (Fig. 6). Choi *et al.* showed that in aqueous solution reduction rates correlate with reduction potentials, and that the rates of reduction of platinum(IV) complexes depend on the bulkiness of the equatorial ligands.[69]

The reduction of Pt(IV) to Pt(II) requires a two-electron transfer process. For Pt(IV) complexes with two halides in *trans* orientation it was suggested that the reduction occurs *via* an attack by the reductant on a coordinated halide.[70] By analogy, it was suggested that the reductive elimination reaction of compounds with two axial acetato ligands, may transpire *via* oxygen-bridge electron transfer.[71]

Most of the studies on the reduction of Pt(IV) complexes were carried out using UV spectroscopy to monitor the disappearance of the Pt(IV) complex. The reduction of Pt(IV) can be monitored by absorption spectroscopy only in the absence of other chromophores that absorb in the same region. Thus, it cannot be used to monitor reduction in biological fluids or in cells, and it does not provide any information on the nature of the reduction products. Hambley and coworkers used

X-ray absorption near edge spectroscopy (XANES) to follow the reduction of Pt(IV) complexes in cells and spheroids.[42,43] There are two major disadvantages to using XANES: (a) the reduction cannot be monitored continuously and a separate experiment has to be conducted to obtain each time point, and (b) the experiments have to be performed in a synchrotron.

Reduction proceeds by elimination of the two axial ligands from the octahedral complex resulting in a square planar platinum(II) complex that retains its original equatorial ligands (Fig. 7). Thus, loss of the two axial acetato ligands from *ctc*-[$PtCl_2(CH_3COO)_2(NH_3)$(Am)] by reductive elimination should lead to the formation of *cis*-[$PtCl_2(NH_3)$(Am)], and two equivalents of free acetate (Fig. 7). Since there is a difference in the proton chemical shifts of the methyl group an acetato ligand that is bound to Pt(IV) and that of the free acetate, NMR spectroscopy can be used to monitor the reduction.[71] We prepared Pt(IV) complexes with ^{13}C, labeled axial acetato ligands with the general formula *ctc*-[$PtCl_2(^{13}CH_3COO)_2(NH_3)$(Am)], in order to monitor the proton chemical shifts of the acetate in biological fluids using [^1H,^{13}C] HSQC NMR spectroscopy.[72]

We used this technique to monitor the rate of reduction of Pt(IV) complexes in whole cell extracts.[72] We found that the rates of reduction for a given compound, *ctc*-[$PtCl_2(^{13}CH_3COO)_2(NH_3)_2$], depend on the nature of the cells that were used. We saw that the half life for *ctc*-[$PtCl_2(^{13}CH_3COO)_2(NH_3)_2$] in A2780cisR is 36 min, in A2780 90 min and in HT-29 130 min. Since there are many reducing agents in the cells, the concentrations and compositions of the various cellular reducing agents are very important in determining the rate of reduction in cell extracts. It is often thought that low molecular mass reducing agents such as ascorbate or glutathione are responsible for the cellular reduction of the Pt(IV) prodrugs. This seems consistent with our observation that reduction proceeded most rapidly in the extracts of the cisplatin resistant cells line (A2780cisR) which has elevated levels of GSH. To the best of our knowledge there is no experimental data implicating specific cellular reducing agents in the reduction of Pt(IV) prodrugs. It seems likely that the reduction of the Pt(IV) prodrugs is carried out by more than one reducing agent.

To assess the role of the low molecular mass cellular antioxidants, including ascorbate and GSH, in the reductions of Pt(IV) complexes, the cell extracts were separated into low and high molecular mass fractions (using a 3 kDa filter), and the ability of each fraction to reduce *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] was measured. While the rate of reduction by the high MW fraction was similar to the rate of reduction exhibited by the whole extracts, the low MW fraction seems to be quite inefficient at reducing the Pt complexes [20% reduction after 500 min]. Our findings, that high molecular mass biomolecules can efficiently reduce Pt(IV) complexes with axial acetato ligands, are consistent with a report by McKeage who showed that metalloproteins, such as cytochrome c and hemoglobin, in the presence of NADH can efficiently reduce satraplatin.[73]

Even more surprising were the results observed for the reduction of *ctc*-[$PtCl_2(^{13}CH_3COO)_2(NH_3)$(NBA)] by ascorbate. Surprisingly, after 10 min of reaction at pH = 7 and 37 °C, three peaks were observed in the [^1H,^{13}C] HSQC spectrum (Fig. 8—top left): one peak corresponds to ctc-[$PtCl_2(^{13}CH_3COO)_2(NH_3)$(NBA)] (NBA = n-butylamine), and another to free acetate, but in addition there is an unexpected peak in the middle. The peak of the starting material decayed quickly, leaving only peaks 2 and 3 in the spectrum (Fig. 8—top right). With time, the middle peak grew weaker with a concomitant enhancement of the free acetate and eventually the only peak observed was that of free acetate (Fig. 8—bottom left). The time course is depicted on the bottom right showing the slow conversion of the middle peak to free acetate. This intermediate (middle) peak was also observed when cytochrome c and NADH were used as the reducing agents. Since the intermediate peak is not free acetate, it must correspond to an acetato ligand that is bound to either Pt(IV) or Pt(II).

In an attempt to identify the products of the reductive elimination, the doubly labeled complex *ctc*-[$PtCl_2(^{13}CH_3COO)_2(^{15}NH_3)$(NBA)] was prepared, and its reduction by ascorbate (in water) was monitored utilizing both [^1H,^{15}N] and [^1H,^{13}C] HSQC NMR spectroscopy.

The ^1H chemical shifts of the $^{15}NH_3$ ligand are very sensitive to the oxidation state of the platinum. For Pt(IV)-$^{15}NH_3$ complexes the proton chemical shifts are around 7 ppm while for the Pt(II)-$^{15}NH_3$ complexes they fall between 3–5 ppm, making it easy to identify the oxidation state. The ^{15}N chemical shifts of the Pt(II)-$^{15}NH_3$ complexes are very sensitive to the to the type of donor that is *trans* to the ammine, and when the ammine is *trans* to a chloride, the ^{15}N chemical shift is around −65 ppm and when *trans* to an oxygen, around −80 ppm. To distinguish between the Pt(II) complexes directly generated by the reductive elimination reaction, and Pt(II) complexes obtained by aquation of the reduced compounds, the experiment was carried out at 10 °C to slow down any substitution reactions (aquation), that might follow the reduction. The [^1H,^{15}N] HSQC spectrum that was obtained after 10 min is depicted in Fig. 9. The peak of *ctc*-[$PtCl_2(^{13}CH_3COO)_2(^{15}NH_3)$(NBA)] appears in the Pt(IV) region, indicating that the reduction was not yet complete. In addition, there are four peaks in the Pt(II) region. Two of them, peaks **a** and **b**, are in the region where the $^{15}NH_3$ is *trans* to a chloride (−65 ppm) and peaks **c** and **d** are in the −80 ppm ^{15}N chemical shift region, indicating that the $^{15}NH_3$ is *trans* to an oxygen. If reduction occurred solely by elimination of the two axial acetato ligands, only one peak would be observed with a ^{15}N chemical shift around −65 ppm corresponding to *cis*-[$PtCl_2(^{15}NH_3)$(NBA)].

These data are not consistent with the notion underlying the design of active Pt(IV) prodrugs—that the sole product of the reductive elimination of *cis*-diam(m)inedichloridoplatinum(IV) complexes with axial acetato ligands are the square planar Pt(II) complexes that retain the original equatorial *cis*-diam(m)inedichlorido coordination sphere. Interestingly, the formation of the intermediates was observed in the reduction of satraplatin by cancer cell extracts. Thus, it seems that reduction of *cis*-diam(m)inedichloridoplatinum(IV) with axial acetato ligands does not proceed solely by loss of two axial ligand and instead of the expected single product in the [^1H,^{15}N] HSQC spectrum, 4 peaks are detected suggesting the existence of multiple reduction pathways.

We are in the process of characterizing the reduction products, but it is clear that formation of several Pt(II) complexes from the reduction of diam(m)inedichloridoplatinum(IV) complexes with axial acetato ligands cannot be reconciled with a single reduction pathway.

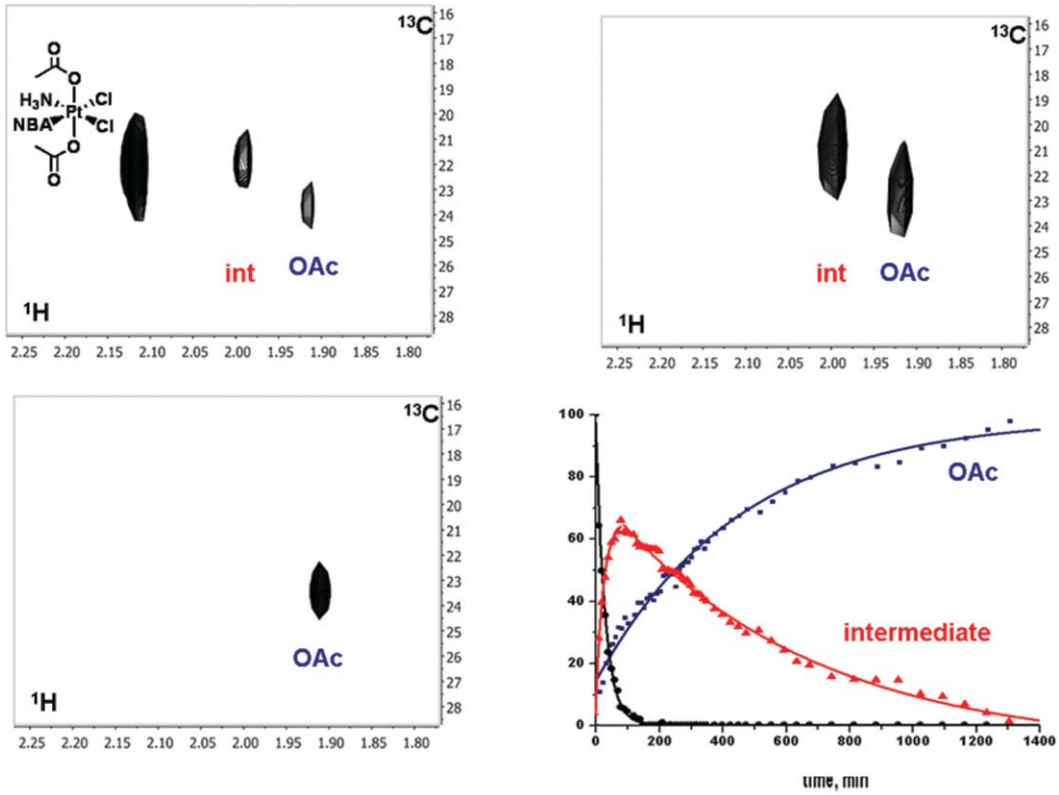

Fig. 8 Reduction of *ctc*-[$PCl_2(OCO^{13}CH_3)_2(NH_3)$(n-butylamine)] monitored by [^1H,^{13}C] HSQC. After 10 min the peak of the starting material, free acetate and an intermediate peak in the middle are visible (top left). With time the starting material disappears and only the peaks of the intermediate and free acetate appear (top right) and finally only free acetate is observed (bottom left). The time course is depicted on the bottom right showing a long lived intermediate that is slowly converted to the acetate.

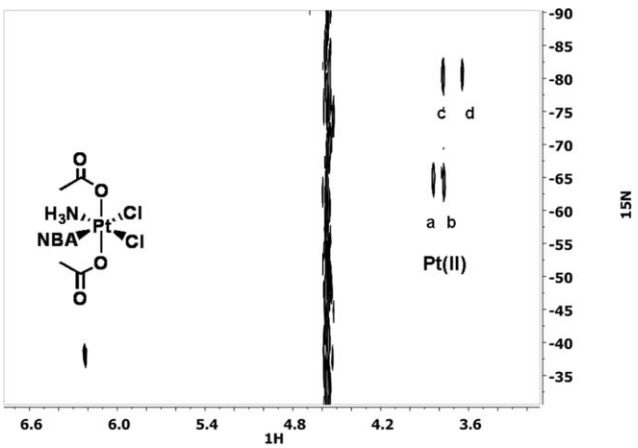

Fig. 9 Reduction of *ctc*-[$PCl_2(OCO^{13}CH_3)_2(^{15}NH_3)$(n-butylamine)] monitored by [^1H,^{15}N] HSQC. After 10 min at 10 °C, the peak of the Pt(IV) starting material and four peaks in the Pt(II) region are visible. Two of the peaks have a chlorido *trans* to the labeled ammine and two have oxygen donors *trans* to the labeled ammine. This suggests that the intermediate observed in the [^1H,^{13}C] HSQC is a Pt(II) complex.

Concluding remarks

We have shown that some of the most common working hypotheses such as the assumption that glutathione is the major cellular target of cisplatin, may need to be re-examined using cutting edge technologies. The need to re-examine the working hypotheses stems primarily from the inability to observe the reactions of Pt complexes in cells and hence we are compelled to adopt model systems that are often too simplistic to accurately predict the events that occur inside the cells. It is important to try and develop methods that can provide the resolution and sensitivity that are necessary to monitor the platinum reactions in biological fluids, rather than work with inadequate model systems simply because they are compatible with the existing technologies. It is the use of 2D inverse detection NMR spectroscopy of labeled complexes that allowed us to measure the half life of cisplatin in cell extracts and compare the rates of reduction of Pt(IV) complexes by extracts from different cells lines, and by different fractions of these cell extracts. This approach also led to the discovery that the mechanism of reduction of *cis*-diam(m)inedichloridoplatinum(IV) with axial acetate ligands may not be as simple as commonly assumed.

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