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Is Glutathione the Major Cellular Target of Cisplatin? A Study of the Interactions of Cisplatin with Cancer Cell Extracts

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Cisplatin is an anticancer drug whose efficacy is limited because tumors develop resistance to the drug. Resistant cells often have elevated levels of cellular glutathione (GSH), believed to be the major cellular target of cisplatin that inactivates the drug by binding to it irreversibly, forming $[Pt(SG)_2]$ adducts. We show by $[^1H,^{15}N]$ HSQC that the half-life of ^{15}N labeled cisplatin in whole cell extracts is ~75 min, but no Pt−GSH adducts were observed. When the low molecular mass fraction (<3 kDa) of the extracts was incubated with cisplatin, binding to GSH was observed probably due to removal of high molecular mass platinophiles. Two-thirds of the Pt adducts formed in whole cell extracts, had a molecular mass >3 kDa. $[Pt(SG)_2]$ cannot account for more than 20% of the Pt adducts. The concentration of reduced thiols in the high molecular mass fraction of the extracts is six times higher than in the low molecular mass fraction.

Introduction

Platinum(II) compounds such as cisplatin, carboplatin, and oxaliplatin (Figure 1), represent an important class of anticancer drugs, from both a clinical and commercial viewpoint.[1−4] Platinum anticancer drugs are believed to kill cancer cells by binding to the nuclear DNA, distorting its structure and thereby triggering cellular processes that result in apoptosis.[5−7] Only a tiny fraction of the cellular platinum binds to the nuclear DNA, which is its critical pharmacological target.[8] Because cisplatin has a much higher affinity to sulfur donors, such as cysteines and methionines, than to the nitrogen donors on the DNA,[9,10] it seems reasonable to assume that most of the cellular platinum is engaged and inactivated by the plethora of sulfur containing cytoplasmatic ligands.[11,12] Not only do non-DNA interactions account for the majority of the cellular adducts formed by cisplatin, but there is also evidence suggesting that these adducts may be responsible for the undesirable side effects of the drug and for development of resistance.[13]

When cancer cells are exposed to cisplatin, over time they can acquire resistance to the drug.[14−17] Acquired resistance has long been recognized as one of the major drawbacks of cisplatin chemotherapy, and many attempts were made to design drugs that circumvent this resistance.[18−21] Often, cells resistant to cisplatin have elevated levels of cellular glutathione (GSH[a]).[22] It is commonly assumed that GSH does play a major role in the mechanism of cellular resistance to cisplatin, although the role that GSH might play in enhancing cellular resistance to cisplatin is still under debate. Picoplatin, *cis*-$[PtCl_2(NH_3)$(2-picoline)], an analogue of cisplatin in which one ammine ligand was replaced by a bulky 2-picoline, was specifically designed to overcome cisplatin resistance by slowing its binding to cellular GSH.[23,24] Some studies found a correlation between the relative ineffectiveness of cisplatin in several ovarian cancer cell lines and elevated cellular levels of GSH,[25] while others concluded, on the basis of a study in 19 cancer cells lines, that there is no correlation between GSH levels and the potency of cisplatin in those cells.[26,27] A recent paper suggests that elevated GSH levels sensitize cells to cisplatin by up-regulating the copper transport hCtr1 receptor that facilitates cisplatin uptake into cells.[28] Cisplatin has a high affinity for sulfur containing ligands and reacts readily with GSH in aqueous solutions under "physiological conditions".[29−31] The GSH concentrations in cells are in the mM range,[26,27] while the intracellular platinum levels are in the μM range; it is thus intuitively appealing to assume that GSH is the major cellular target of cisplatin and that the reaction between them leads to stable covalent adducts which prevent the platinum from binding to DNA (Figure 2). [11]

Ishikawa and Ali-Osman studied the interactions of cisplatin with L1210 murine leukemia cells and concluded that after a 12 h incubation, 60% of the intracellular cisplatin reacted with GSH, resulting in the displacement of both ammine ligands to form the bis-(glutathionato)-platinum, $[Pt(GS)_2]$, which has a molecular weight of 809 Da (complex D Figure 2).[32] Interestingly, when Berners-Price and Kuchel studied the reactions of cisplatin with reduced GSH in red blood cells by NMR, they were unable to detect Pt−GSH bonds.[33] They did notice that following the incubation of the red blood cells with cisplatin, the GSH levels in the low

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[a] Abbreviations: GSH, glutathione; HSQC, heteronuclear single quantum correlation; Met, methionine; Cys, cysteine; DDW, doubly distilled water; SEC, size exclusion column; HSAB, hard soft acid base; PBS, phosphate buffered saline; FCS, fetal calf serum; MWCO, molecular weight cutoff; DTNB, 5,5′-Dithio-bis(2-nitrobenzoic acid); NAC, N-acetylcysteine.

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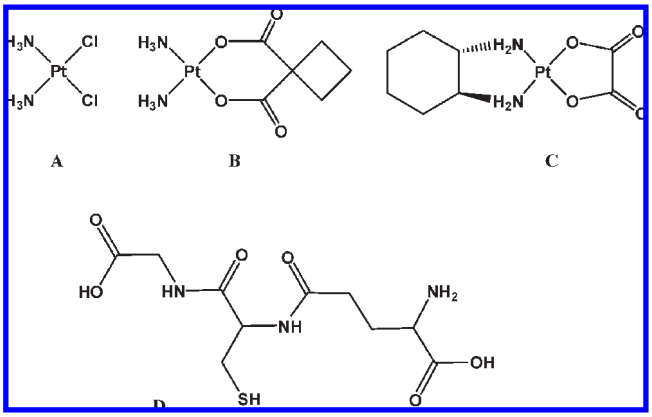

Figure 1. Three clinically used Pt anticancer agents: (A) cisplatin, (B) carboplatin, (C) oxaliplatin, and (D) the thiol containing tripeptide GSH.

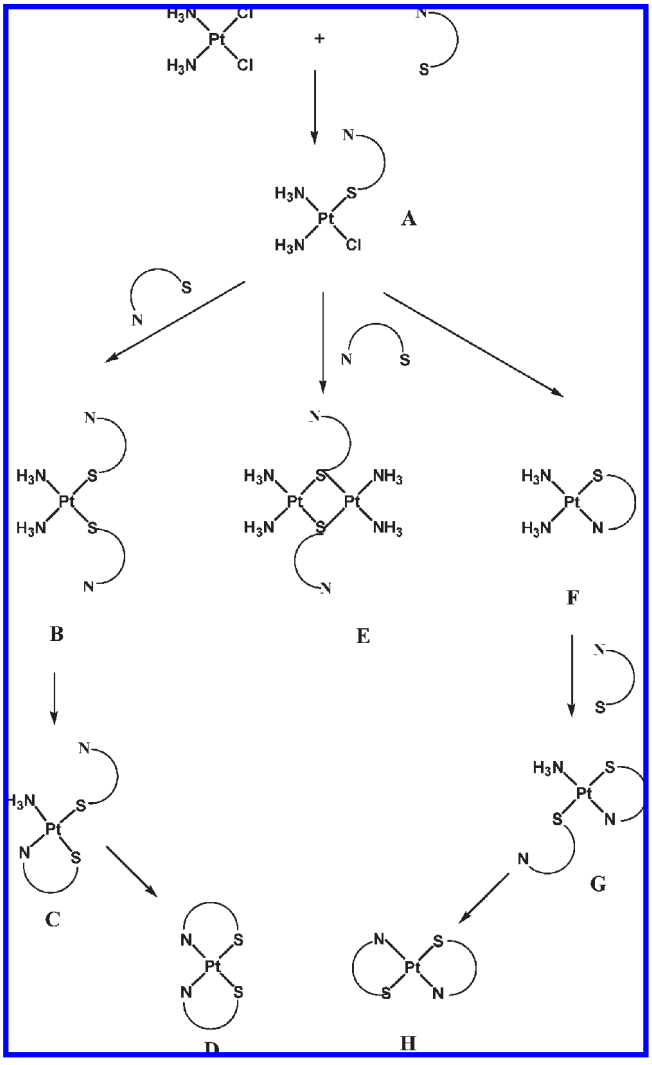

Figure 2. Some of the possible products of the reaction of cisplatin with excess GSH. All species, with the exception of D and H, should be detectable by [^{1}H,^{15}N] HSQC NMR.

molecular mass fraction of the cell extracts (<10 kDa) decreased by 60%. The absence of Pt−GSH peaks in the T2 dependent NMR experiments and the dramatic drop in GSH levels led them to conclude that the Pt bound GSH was present in complexes with molecular masses higher than 10 kDa. They have not, however, directly identified or characterized ternary protein−Pt−GSH complexes. They also observed some free ammine ligand after a 12 h incubation, indicating that some trans-labilization occurred.[33]

In a recent paper, *Escherichia coli* cells were treated with cisplatin and multidimensional liquid chromatography and electrospray ionization tandem mass spectrometry were used to identify 31 proteins to which Pt was bound.[34] These included high abundance enzymes and ribosomal proteins as well as DNA and RNA binding proteins. Contrary to expectation, based on the HSAB (hard soft acid base) theory,[35] 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 proteins and methionine was identified as the binding site only nine times. Cisplatin binding to these high molecular mass proteins is in agreement with the observations by Berners-Price and Kuchel.[33] However, the mass spectral analysis reveals 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. The fact that in some cases only $[Pt]^{2+}$ was bound to a protein implies that some trans-labilization of ammine ligands occurred.

The conflicting nature of the reports regarding the formation of Pt−GSH adducts upon incubation of cells with cisplatin, coupled with the general lack of understanding of how, or if, GSH is involved in sequestering the cellular activity of cisplatin, prompted us to take a closer look at the interactions of cisplatin with aqueous extracts of cancer cells.

Results

One of the biggest challenges in elucidating the mechanism of action of cisplatin is finding ways to continuously monitor and identify the various platinum species that form inside proliferating cells. It is widely accepted that once cisplatin enters the cytoplasm it undergoes aquation and hydrolysis to form primarily *cis*-$[PtCl(H_2O)(NH_3)_2]^+$ and *cis*-$[Pt(H_2O)_2(NH_3)_2]^{2+}$, which proceed to react with the DNA and possibly other nucleophiles.[7,8] Other reports suggest that cisplatin forms carbonato complexes as well as complexes with phosphates and acetates and especially with GSH.[32,36−38] Unfortunately, there are no experimental methods that can monitor platinum speciation at μM concentrations inside cells or even obtain the half-life of cisplatin in the cytoplasm. Consequently, the cellular fate of cisplatin is often deduced from extrapolation from the reaction chemistry of cisplatin with low molecular mass biological nucleophiles in buffered aqueous solutions.[39−42]

One of the few methods that can continuously and noninvasively monitor the reactions of cisplatin is 2D NMR spectroscopy, [^{1}H,^{15}N] HSQC, of ^{15}N labeled cisplatin.[43] Because it is not possible to monitor the reactions of cisplatin inside cells, we decided to use aqueous extracts of cancer cells as models for the intracellular environment (cytoplasm). ^{15}N labeled cisplatin was added to the cancer cell extracts or to different fractions of the extracts (high and low molecular mass fractions and anionic and cationic fractions), and the fate of cisplatin was monitored by [^{1}H,^{15}N] HSQC NMR spectroscopy. This technique is ideally suited for determining the half-life of cisplatin in biological fluids and for detecting the expected aquation products, carbonate or phosphate binding, and binding to sulfur containing ligands.[33] Since it

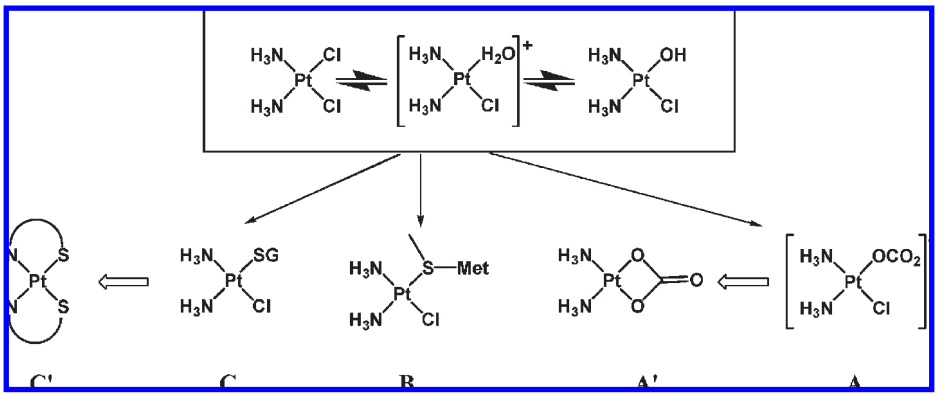

Figure 3. Some of the cisplatin metabolites that might be found inside cells. Cisplatin and its monoaquated and hydrolyzed transformation (in the box), which can react with carbonates to form A that transforms to A', or with methionine (B), which can react further. Of special interest is the reaction with GSH, which begins with the formation of C that may transform to C′ after several stages. All species, with the exception of C′, should be detectable by [^{1}H,^{15}N] HSQC.

was reported that 60% of the cellular cisplatin binds to GSH, we expected to be able to observe these interactions in cell extracts by [^{1}H,^{15}N] HSQC. We therefore began with a control experiment in which we used [^{1}H,^{15}N] HSQC NMR spectroscopy to study the interactions of cisplatin with a 10-fold excess of GSH in order to identify the signature peaks of the cisplatin−GSH adducts (Figure 3), which could later be correlated to peaks that form when cisplatin is incubated with cell extracts or fractions of the extracts. This experiment was also designed to check whether GSH will rapidly trans-labilize the labeled ammine ligands of cisplatin and result in the loss of the HSQC signals.[44]

Control Experiment: Monitoring the Reactions of *cis*-[PtCl$_2$(^{15}NH$_3$)$_2$] with excess GSH, by [^{1}H,^{15}N] HSQC. The ^{15}N chemical shift of the ^{15}NH$_3$ ligand of *cis*-[PtCl$_2$(^{15}NH$_3$)$_2$] is very sensitive to the nature of the ligand trans to it, and in case of oxygen, it resonates around −85 ppm, when trans to chloride or nitrogen around −65 ppm and when trans to sulfur around −40 ppm.[43] C*is*-[PtCl$_2$(^{15}NH$_3$)$_2$], 1 mM, was incubated with 10 mM GSH in water (pH = 7) at 37 °C. The [^{1}H,^{15}N] HSQC spectra were collected every 10 min for the first 100 min, every hour for the next 2 h, and every 2 h thereafter. Peaks due to formation of H$_3$^{15}N−Pt-S adducts began to appear after 10 min, and several strong peaks were observed even after 13 h, indicating that the labeled ammine ligands were not rapidly displaced by GSH binding. GSH can act as a monodentate ligand that binds to the platinum via the cysteine thiolate but can also act as a bidentate ligand by binding the platinum through both the thiol and the amide nitrogen. The first step in the reactions of GSH with cisplatin is the displacement of the chloride ligand by the thiolate of cysteine, forming an adduct (Figure 2, compound A) that should be easily detected in the [^{1}H,^{15}N] HSQC spectra. The ability of GSH to form a chelate with Pt(II) affords a variety of possible products that are schematically depicted in Figure 2. With the exception of the bis-(glutathionato)-platinum complexes (compounds D and H in Figure 2), all the other platinum−GSH complexes depicted in Figure 2 should give rise to peaks in the [^{1}H,^{15}N] HSQC spectra, most of them in the −40 ppm region in the ^{15}N dimension. Trans-labilization of the ^{15}N labeled ammine would result in the loss of signal due to the rapid exchange of the hydrogen atoms of the free ^{15}N labeled ammine with the water at neutral pH. Figure 4 shows the [^{1}H,^{15}N] HSQC spectra of the 1:10 cisplatin:GSH reaction mixture at various time points. Significantly, not all of the cisplatin was converted to bis-(glutathionato)-platinum, [Pt(GS)$_2$], which is HSQC silent. Therefore, we believe it should be possible to detect Pt−GSH adducts in cancer cell extracts.

[^{1}H,^{15}N] HSQC Studies of the Reactions *cis*-[PtCl$_2$(^{15}NH$_3$)$_2$] with Cancer Cell Extracts. The pH values of the NMR samples of the cell extracts were measured and ranged from 6.8 to 7.2 and their GSH levels were approximately 2 mM. *cis*-[PtCl$_2$(^{15}NH$_3$)$_2$] was added to the extracts so that the final concentration of cisplatin in the NMR samples was 250 μM. The reactions were monitored by [^{1}H,^{15}N] HSQC at 37 °C. On the basis of the presumed mechanism of action of cisplatin, we hoped to see peaks in the [^{1}H,^{15}N] HSQC in the −80 ppm ^{15}N chemical shift region due to aquation, binding to carbonates or phosphates. Furthermore, we also expected to see strong peaks in the −40 ppm region due to binding to GSH (Figure 3). Contrary to our expectations, we did not observe the formation of ^{15}NH$_3$−Pt-S bonds (HSQC peaks in the −40 ppm ^{15}N chemical shift region). The only peak in the [^{1}H,^{15}N] HSQC spectra was that of cisplatin at (4.0,−65) ppm (the spectra look that the one depicted in Figure 6C), which became weaker as time went by until it completely disappeared after ∼300 min with a $t_{1/2}$ of 75 min. (see Figure 5). It is possible that aquated species such as *cis*-[PtCl(H$_2$O)(NH$_3$)$_2$]$^+$ were formed, but due to their reactivity, their steady state concentration was too low to detect. There are several possible reasons as to why the expected binding to sulfur containing nucleophiles was not observed: (a) there are many sulfur containing nucleophiles in the cell extracts, making it possible for cisplatin to form a large number of adducts so that the concentration of each may be too low to be detected by a 500 MHz NMR spectrometer, (b) if cisplatin binds to high molecular mass nucleophiles (even to Met or Cys residues on them), the slow rotational correlation time of these conjugates will broaden the HSQC signals to the point where they will no longer be detected, (c) if upon binding to sulfur containing nucleophiles there is rapid trans-labilization of the ^{15}NH$_3$ ligands, the HSQC signal will be lost. To improve our chances of detecting low levels of adducts, we repeated the [^{1}H,^{15}N] HSQC experiments using a 900 MHz NMR spectrometer equipped with an inverse detection triple resonance cryoprobe. Despite the high sensitivity of the 900 MHz spectrometer, no binding to sulfur containing ligands was observed. Again, the only peak observed was that of cisplatin. The decay curves of cisplatin in cell extracts, as measured on both spectrometers, are the same, yielding an

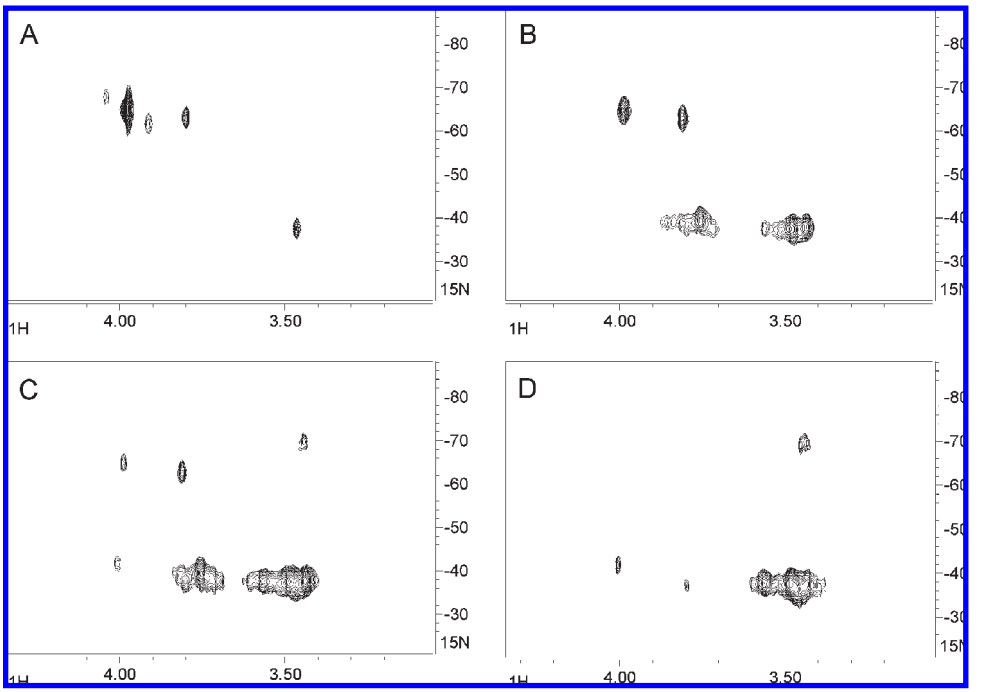

Figure 4. [^{1}H,^{15}N] HSQC NMR spectra of the reaction between 1 mM cisplatin and 10 mM GSH at 37 °C. (A) after 50 min, (B) after 160 min, (C) after 380 min, and (D) after 820 min.

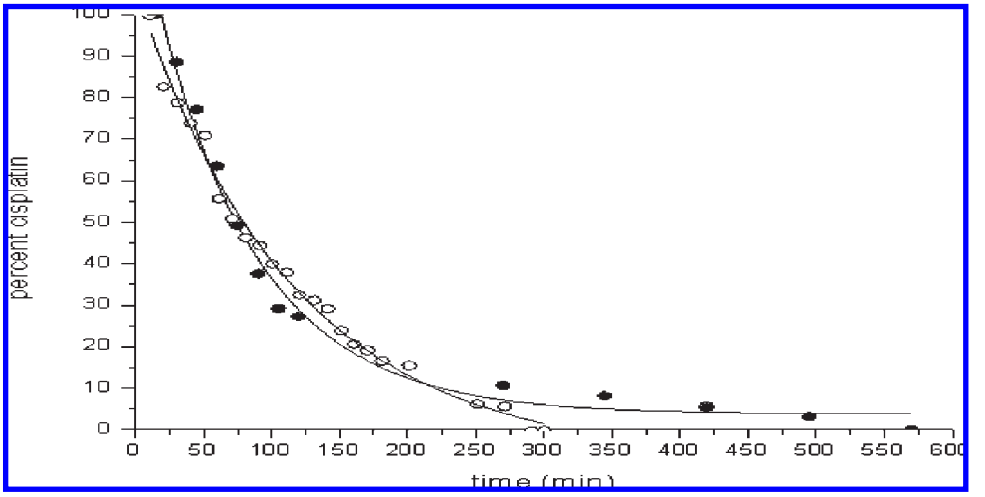

Figure 5. Decay curves of ^{15}N labeled cisplatin HSQC signal in cancer cell extracts at 37 °C as measured by 500 and 900 MHz NMR spectrometers, yielding a $t_{50\%}$ of approximately 75 min.

identical half-life of ~75 min. For cisplatin to bind to DNA, its critical pharmacological target, one of the chloride ligands must be substituted by a water molecule to form *cis*-[PtCl-$(H_2O)(NH_3)_2]^+$.[7] The half-life for the formation of *cis*-[PtCl-$(H_2O)(NH_3)_2]^+$ is 120 min,[6] which is significantly longer than the half-life of cisplatin in the cell extracts. To the best of our knowledge, this is the first estimate of the half-life of cisplatin in the cytoplasm of cancer cells.

[^{1}H,^{15}N] HSQC Studies of the Reactions *cis*-[$PtCl_2(^{15}NH_3)_2$] with Low and High Molecular Mass Fractions of Cancer Cell Extracts. To check the extent and rate of cisplatin binding to high molecular mass nucleophiles in cell extracts, we performed two types of experiments: (a) cisplatin was incubated with whole cell extracts and then the reaction mixture was separated into fractions according to molecular mass, and the platinum was quantified in each fraction by ICP-MS (see below), and (b) the cell extracts were separated into the high and low molecular mass fractions and each fraction was incubated with *cis*-[$PtCl_2(^{15}NH_3)_2$], and the interactions were monitored by [^{1}H,^{15}N] HSQC. The levels of GSH in both the low and high molecular mass fractions (separated by centricons with MWCO of 3 kDa) were quantified, and 95% of the reduced GSH was found in the low molecular mass fraction, suggesting it is available for Pt binding. When *cis*-[$PtCl_2(^{15}NH_3)_2$] was incubated with the low molecular mass fraction of the cell extracts, several peaks were observed in the HSQC spectrum in addition to the peak of the starting material. There were peaks in the −60 ppm region, indicative of formation of adducts with nitrogen donors trans to the labeled ammine ($^{15}NH_3$−Pt-N), as well as peaks in the −40 ppm region, which are typical of the formation of adducts with a sulfur donor trans to the labeled ammine

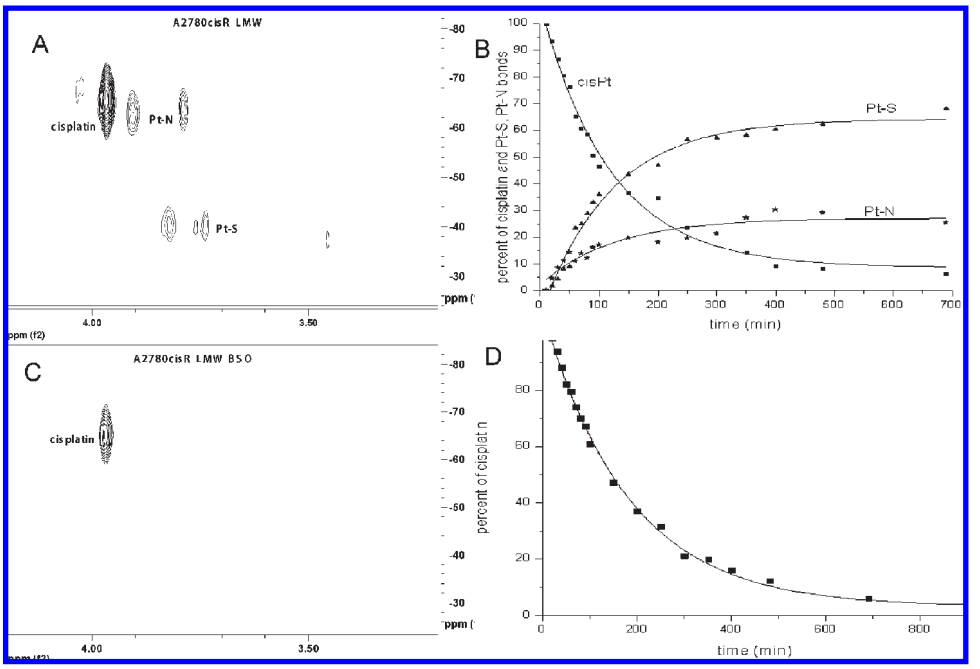

Figure 6. Reactions of cisplatin with the low molecular mass fraction of extracts from A2780cisR cells: (A) The [^{1}H,^{15}N] HSQC spectrum showing peaks that are attributed to binding to sulfur and to nitrogen donors, (B) the relative ratios among the different types of adducts obtained from integration of the HSQC spectra. The squares show the decay of cisplatin, the stars show the formation of ^{15}N−Pt−N adducts and triangles the formation of ^{15}N−Pt−S adducts. The same information is displayed for cells that we preincubated with BSO: (C) the HSQC spectrum and (D) the decay curve of cisplatin.

($^{15}NH_3$−Pt-S). (Figure 6A) After 400 min of incubation, the $^{15}NH_3$−Pt-S peaks comprised ∼60% of the all the signals in the [^{1}H,^{15}N] HSQC spectrum and the $^{15}NH_3$−Pt-N peaks 30%, with only 10% belonging to the starting material. (Figure 6B). Interestingly, no peaks denoting an oxygen donor trans to the labeled ammine were observed. The $t_{1/2}$ of cisplatin in the low molecular mass fraction was ∼90 min, which is higher than that obtained with the whole cell extracts yet lower than that required for the first aquation of cisplatin. When cisplatin was incubated with the high molecular mass fraction, only the peak of the starting material was observed, as in the case of the whole cell extracts, and decayed with a $t_{1/2}$ of ∼75 min.

To determine whether the $^{15}NH_3$−Pt-S HSQC peaks observed when cisplatin was incubated with the low molecular mass fraction originated from reactions with GSH, a control experiment was conducted with extracts in which GSH levels were drastically reduced (by a factor of 14) by preincubating the cells with BSO (a specific inhibitor of GSH biosynthesis). When the low molecular mass fraction of the GSH depleted extract was incubated with *cis*-[$PtCl_2(^{15}NH_3)_2$], only the peak of the starting material was observed (Figure 6C). This suggests that the $^{15}NH_3$−Pt-S peaks resulting from the reactions of cisplatin with the low molecular mass fractions of normally grown cells are indeed due to binding to GSH. The $t_{1/2}$ of cisplatin in the GSH depleted low molecular mass fractions was nearly 50% longer (125 min) than in the normal low molecular mass fraction (Figure 6D), lending further support to the suggestion that GSH is the primary target of cisplatin when its is incubated with the low molecular mass fraction of the extracts.

[^{1}H,^{15}N] HSQC Studies of the Reactions of *cis*-[$PtCl_2(^{15}NH_3)_2$] with Cationic and Anionic Fractions of Cancer Cell Extracts. Whole cell extracts were passed through a strong cation exchanger in the sodium form and washed with water. *cis*-[$PtCl_2(^{15}NH_3)_2$] was incubated with the eluate containing the anions and neutral molecules of the original extracts (the anionic fraction from hereon), and the reactions were monitored by [^{1}H,^{15}N] HSQC. Similarly, a cationic fraction was generated (by passing the extracts through an anion exchanger) and its reactions with *cis*-[$PtCl_2(^{15}NH_3)_2$] were monitored by [^{1}H,^{15}N] HSQC.

In the reaction of the anionic fraction with *cis*-[$PtCl_2(^{15}NH_3)_2$], peaks indicative of sulfur binding were observed, while in the reactions of the cationic fraction with *cis*-[$PtCl_2(^{15}NH_3)_2$], only the peak of the starting material was detected.

These results are reminiscent of the differences observed between the interactions of cisplatin with the low and high molecular mass fractions, which were attributed mainly to the reactions of cisplatin with GSH in the former. At neutral pH, GSH is an anion and will only be present in the anionic fraction. It is interesting to note that the half-life of cisplatin in the cationic fraction ($t_{1/2}$ = 65 min) is significantly shorter than in the anionic fraction ($t_{1/2}$ = 140 min), even though the sulfur containing adducts (detected by HSQC) in the anionic fraction account for 70% of the observed species after 400 min.

Quantification of the Adducts that *cis*-[$PtCl_2(NH_3)_2$] Forms with the Different Fractions of the Aqueous Extracts of Cancer Cells. The results of the [^{1}H,^{15}N] HSQC studies described above prompted us to examine the size distribution of the covalent platinum adducts formed by the incubation of cisplatin with cell extracts and to see if 60% of the cisplatin does indeed end up in the form of [$Pt(SG)_2$]. Whole cell extracts were incubated with cisplatin and allowed to react overnight. In the HSQC experiments, each extract was prepared from 160 million cells in 250 μL and incubated with 250 μM cisplatin. Because ICP-MS is a very sensitive

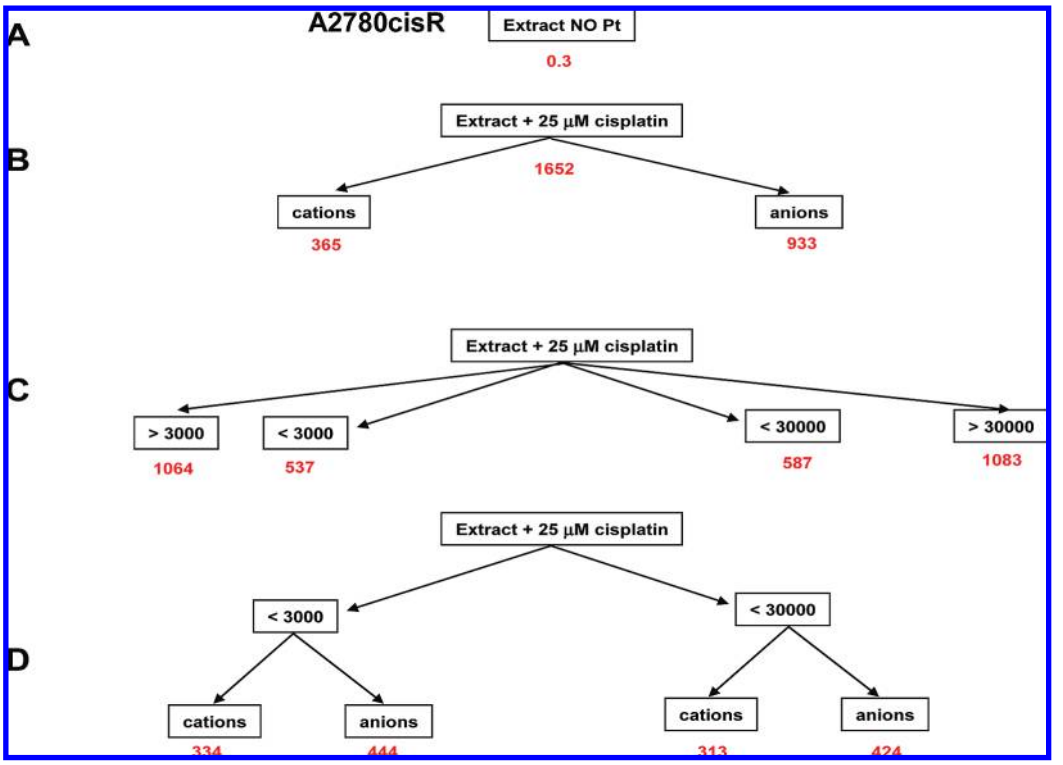

Figure 7. Depiction of the fractionations that were carried out on the A2780cisR cell extracts that were incubated with cisplatin in order to quantify the Pt levels in each fraction. The numbers under the boxes are the Pt levels in ng. (A) control, no cisplatin was added to the extracts, (B) cationic vs anionic adducts formed by cisplatin, (C) quantification of extracts that were separated to low and high molecular mass by centricons (3 and 30 kDa), (D) quantification of Pt in the cationic and anionic subfractions of the low and high molecular mass fractions of the extracts.

technique, for the quantification experiments we used extracts from 16 million cells in 250 μL and adjusted the cisplatin concentration to 25 μM in order to maintain the same Pt:cell ratio as in the HSQC experiments. After the incubation, the extracts were separated into low and high molecular mass fractions using membranes with molecular weight cutoffs of either 3 or 30 kDa. The platinum content in each of the fractions was determined by ICP-MS. The fractionation procedures and the results of the platinum quantification in each fraction (ng) are detailed in Figure 7. We chose to carry out the platinum quantification experiments on cisplatin that was incubated with cell extracts, rather than with proliferating cells, in order to be able to compare the analytical results with those of the HSQC studies.

We found that 65% of the platinum is bound to cellular nucleophiles with a molecular mass greater than 3000 Da. It is therefore unlikely that bis-(glutathionato)-platinum, [Pt$(GS)_2$], whose molecular mass is 809 Da, accounts for ~60% of the Pt adducts that results from the incubation of cisplatin with cell extracts. Moreover, if ~65% of the Pt is bound to nucleophiles with molecular mass greater than 30 kDa (Figure 7), it is not surprising that they were not observed by [^{1}H,^{15}N] HSQC. [Pt$(GS)_2$] has a molecular mass of less than 3 kDa and is anionic at neutral pH. Therefore, when the extracts are separated into high and low molecular mass fractions with a 3 kDa MWCO, [Pt$(GS)_2$] should be present in the low molecular mass fraction. When the low molecular mass is separated into anionic and cationic fractions, it will be present in the anionic fraction. Only ~57% of the platinum adducts in the low molecular mass fraction were anionic. Even if we assume that all the platinum in the anionic component of the low molecular mass fraction was

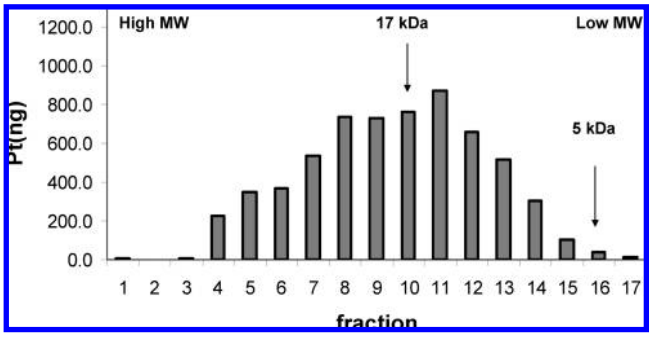

Figure 8. Pt levels (ng) in the fractions obtained from eluting the extracts of A2780cisR cells, which were reacted with cisplatin through the size exclusion column. The lower fractions represent high MW compounds. Fraction 10 contains molecule of ~17 kDa and fraction 16 compounds of ~5 kDa.

in the form of [Pt$(GS)_2$], it would still account for at most 20% of the total platinum content.

To verify that cisplatin forms high molecular mass adducts, cisplatin (25 μM) was incubated overnight with extracts of 16 million A2780cisR cells, and the reaction mixtures were chromatographed on a size exclusion column (SEC), fractions were collected, and their platinum content was quantified by ICP-MS. The results indicate that the molecular masses of the platinum adducts span a fairly broad distribution and that 60% of the platinum adducts that eluted from the SEC column have molecular masses greater than ~20 kDa (Figure 8).

Quantification of Thiols in the Different Fractions of the Aqueous Extracts of Cancer Cells. The hard−soft acid−base theory teaches us that Pt(II) has a high affinity for thiols, and

Table 1. Determination of Thiol Levels in Fractions of Cell Extracts

cell line/fraction	thiol conc (mM)
A2780- LMW	1.02 ± 0.23
A2780cisR-LMW	3.53 ± 0.51
A2780-HMW	21.43 ± 4.11
A2780cisR-HMW	20.55 ± 1.34

although no binding to sulfur was observed by [^{1}H,^{15}N] HSQC when cisplatin was incubated with the high molecular mass fractions, the ICP-MS data indicate that most of the platinum is in the form of high molecular mass adducts. Because the lack of peaks in the HSQC spectra of high molecular mass nucleophiles cannot rule out the formation of Pt−S adducts and because some trans-labilization of the ammine ligands (observed by us and by others)[29,33,44] suggests that some binding to sulfur containing ligands occurred, we decided to quantify the reduced thiols in each fraction to see if there is any correlation between thiol levels and the formation of Pt adducts. Using Ellman's reagent for thiol quantification,[45] we determined the concentrations of thiols in the high and low molecular mass fractions and found that the thiol level in the high molecular mass fraction is approximately 6 times higher than in the low MW fraction. The data appear in Table 1.

Discussion and Conclusions

Elevated GSH levels have often been reported in cancer cells that possess resistance to cisplatin. There are conflicting views in the literature as to whether there is a direct correlation between the degree of resistance of cancer cells to cisplatin and the intracellular GSH levels. Even in cancer cell lines that are sensitive to cisplatin, there is a huge excess (>500-fold) of intracellular GSH relative to the intracellular concentration of cisplatin that could efficiently inactivate cisplatin. Therefore, is the extra GSH in resistant cell lines really necessary to inactivate cisplatin by binding to it? GSH reacts readily with cisplatin in aqueous solution, and thus it seems logical to conclude that most of the intracellular platinum is inactivated as a result of direct binding of GSH to cisplatin.[11,32] On the basis of the report that 60% of the cisplatin reacts with cellular GSH to form primarily a single product, [Pt(GS)$_2$], we expected to observe by [^{1}H,^{15}N] HSQC the Pt−GSH adducts that are the intermediates in the formation of [Pt(GS)$_2$] (Figure 2) when ^{15}N labeled cisplatin was incubated with cancer cell extracts. Even with a very powerful NMR spectrometer (900 MHz), we were not able to see the expected interactions.

Although we cannot identify the adducts that were formed when cisplatin was incubated with the cell extracts, the half-life of cisplatin in the extracts is around 75 min, which is shorter than the half-life for the first aquation of cisplatin.[7] While methionines and cysteines can react directly with cisplatin, substituting the chloro ligands binding to nitrogen and oxygen donors (such as DNA, phosphates, and carbonates) requires initial aquation of cisplatin. Also interesting, and perhaps counterintuitive, is the fact that the half-life of cisplatin in the high molecular mass fraction of the extracts is shorter than in the low molecular mass fraction (75 vs 90 min). This might be due to the higher number of thiols in the high molecular mass fraction.

Inside cells, or in cell extracts, there are many nucleophiles in addition to GSH that can react with cisplatin, complicating the ability to detect Pt−GSH interactions. Cisplatin can initially react with non-GSH cellular nucleophiles, forming stable adducts that can then react with GSH to form Pt mediated ternary complexes. Conversely, cisplatin may react with one molecule of GSH, and this adduct may proceed to react with other cellular nucleophiles. It is quite possible that in addition to 1:1 or 1:2 Pt:GSH adducts, there may be many ternary complexes that contain the Pt−SG moiety. Interestingly, we observed earlier that when adducts of ubiquitin with cisplatin and transplatin are exposed to an excess of GSH, ternary Ub−Pt−GS complexes are formed and slowly the Ub−Pt bonds are severed, resulting in Pt−GSH adducts.[46] Therefore, it is neither an easy nor a straightforward task to quantify the level of Pt−GSH adducts in cells or in cell extracts.

Interestingly, when the GSH containing fractions of the extracts (low MW and anionic) were isolated and incubated with cisplatin, formation of Pt−S bonds was observed and comparison of the interactions of cisplatin with the same fractions from BSO treated cells indicated that the observed Pt−S bonds were indeed due to formation of Pt−GSH adducts. Cisplatin does form adducts with GSH both in aqueous solution and in selected fractions of the cell extracts. Selection of the anionic and/or low molecular mass fractions probably removes many potential platinophiles that compete with GSH for the cisplatin, enabling the formation of Pt−GSH adducts.

The ICP-MS results indicate that two-thirds of the Pt is covalently attached to higher molecular mass nucleophiles (>3000 Da) and are in direct agreement with the results report by Berners-Price and Kuchel.[33] We cannot rule out that the high molecular mass adducts may contain Pt-SG moieties. The analytical data obtained here does not support the claim that 60% of the cisplatin adducts is in the form of the bis (glutathionato) complex, [Pt(SG)$_2$], because this adduct would be expected to be in the anionic part of the low MW fraction. Our data show that even if all the Pt in the low molecular mass anionic fraction were due to [Pt(SG)$_2$], it would account for around 20% of the Pt adducts. Moreover, 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. We quantified the trans-labilization of ammines from cisplatin by cells and cell extracts and were only able to observe 20% trans-labilization after 12 h.[44,47] The results obtained in this study are different from those reported by Ishikawa and Ali-Osman,[32] 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 their results were obtained from intact cells.

The higher levels of GSH in resistant cell lines do not necessarily imply direct reaction between cisplatin and GSH and formation of Pt−GSH adducts. In an attempt to explain why increase in GSH levels, associated with increased resistance, 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.[3,7,48] In another study, reducing cellular GSH levels with BSO did not affect the resistance of the cells, leading the authors to conclude that although exposure of cells to cisplatin results in higher GSH levels, this is not directly involved in cisplatin resistance.[49] 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.[28]

Another noteworthy point pertains to the use of model systems for predicting the cellular behavior of Pt drugs. Cells are quite complex, containing many types of nucleophiles, therefore, using a buffered aqueous solution with one or two added nucleophiles is not likely to be a good predictor of the cellular chemistry. We think that aqueous extracts of cells may be a reasonable model for the cytoplasm, but even different fractions of the same cell extracts displayed different reactivity toward cisplatin, indicating that modeling cellular interactions is not a simple task, especially given that different cell lines react differently with Pt complexes. We believe that it still remains to be seen if and how GSH is involved in the cellular resistance mechanisms to Pt drugs. We do think that the results of this study suggest that direct binding of GSH to cisplatin and formation of binary 1:1 or 1:2 adducts is not the most important or prominent cellular interaction that leads to the inactivation of 60% of the cellular cisplatin.

Experimental Section

Materials. $AgNO_3$, GSH, 5,5′-Dithiobis(2-nitrobenzoin acid), 99%, *N*-acetyl cystein, EDTA, Tris-Base, BSO, D_2O, and PBS were purchased from Sigma-Aldrich, Israel. $^{15}NH_3NO_3$ was purchased from Cambridge Laboratories Isotopes, Nes-Ziona, Israel. FCS, Penstrep and Glutamine were purchased from Biet Haemek, Biological Industries. Medium: RPMI 1640 was purchased from Renium Ltd., Jerusalem. All were used as received without further purification. *cis*-[Pt($^{15}NH_2$)$_2$Cl$_2$], was prepared as previously described.[44,47]

NMR Spectroscopy. All inverse detection NMR spectra for the studies of the interaction of the model compounds with the Pt complexes were recorded using a Varian Unity Inova 500 MHz spectrometer (^{1}H 499.78 MHz, ^{15}N 50.64 MHz) equipped with 5 mm triple resonance inverse-detection probe. The two-dimensional [^{1}H,^{15}N] gradient HSQC NMR spectra were obtained using VNMR pulse sequences and were optimized for ^1J (^{15}N,^{1}H) = 73 Hz. Samples were not spun during acquisition of data. All samples were run in 90% H_2O/10% D_2O) at 37 °C. Typical acquisition parameters include an acquisition time of 0.2 s, 12 transients, a recycle delay of 1.3 s, and 16 increments in the second dimension. The data were processed with either VNMR or MestReC, using sine squared apodization in both dimensions and linear prediction in the second dimension.

Sample Preparation for the NMR Studies. The samples for the reactions of 1:10 *cis*-[Pt($^{15}NH_3$)$_2$Cl$_2$] with reduced GSH were prepared as follows: ^{15}N cisplatin (0.18 mg) was dissolved in 60 μL of 90% H_2O:10% D_2O to form a 1 mM solution, and after complete dissolution of the platinum complex, reduced GSH (1.8 mg, 6 μmol) was added and the reaction mixture was transferred to a 5 mm NMR tube and was immediately inserted into the NMR probe and the experiment was commenced.

The samples for the [^{1}H,^{15}N] HSQC studies with cell extracts were prepared as follows: to the whole extracts or to the fractions (from160 million cells) in 225 μL of DDW, 25 μL of 0.25 mM of ^{15}N labeled cisplatin (0.02 mg, 0.06 μmol dissolved in of D_2O) was added. The samples were transferred to a Shigemi NMR tube and were immediately inserted into the NMR probe and heated to 37 °C.

Preparation of Whole Cell Extracts for NMR Studies. Human ovarian cancer cisplatin resistant A2780cisR cell line (obtained from ECACC) was maintained in RPMI 1640 medium supplemented with 10% FCS, antibiotics, and glutamine. All culture components were purchased from Biological Industries (Beit-HaEmek, Israel). All cell lines were maintained at 37 °C in 5% CO_2/95% air incubator.

The cells were harvested when they reached 70% confluence. The medium was removed, and the cells were washed with 5 mL of cold PBS. The PBS was removed, 3 mL of trypsin were added, and the dish was placed in the incubator. After 3 min, it was taken out and 4 mL of medium were added to the cells. The cells were transferred to a tube and centrifuged for 5 min at 1200 rpm. The trypsin containing medium was removed, and 10 mL of fresh medium was added to the cells. The cells were suspended and counted. They were centrifuged for 5 min at 1200 rpm, and the medium was removed and suspended in 5 mL of PBS. This procedure was repeated twice. The PBS was removed to yield the pellet.

Cell extracts were prepared by lysing 80 million cells in 0.9 mL of deionized water, 1 min vortex, 1 min in ice, three times followed by centrifugation to get rid of the insoluble material and removal of the solvent using a SpeedVac. Then 225 μL of DDW were added to the dry extract, and to that 25 μL of cisplatin (0.2 mg in 250 μL D_2O) was added. The final NMR sample was made of 250 μL of 90% DDW and 10% D_2O containing extracts from 160 million cells and a final cisplatin concentration of 250 μM.

Fractionation of the Cell Extracts. To obtain the low molecular mass of the extract, it was transferred to a centricon (YM-3) with cut off of 3000 Da and was centrifuged at 14000*g* for 200 min at 4 °C. Then 200 μL of DDW was added and centrifugation continued for another hour and the low molecular mass fraction was collected. To obtain the high molecular mass fraction, the centricon was turned up side down and fitted with a new Eppendorf and then centrifuged for 3 min, at 3000*g* and 4 °C. Finally, 50 μL of DDW was added and centrifugation continued for another 3 min and the high molecular mass fraction was collected.

For the SEC study, extracts (80×10^6 cells) were incubated with 250 μM cisplatin for 12 h at 37 °C. Samples were applied to a Superdex 75 analytical column 30 cm × 1 cm (GE Healthcare) using an AKTA Explorer System (GE Healthcare) at 4 °C, equilibrated with buffer: 20 mM Tris pH 8.0 + 50 mM $NaNO_3$. Isocratic elution with the same buffer at 1 mL/min collecting fractions of 1 mL and monitored by absorbance at 280 and 260 nm. The fractions were then submitted to ICP-MS analysis.

To obtain the anionic fraction of the extracts, the whole cell extracts (obtained as described above) were passed through a cation exchange Dowex 50W-X8 (particle size 100−200 mesh) columns in the NH_4^+ form and washed with DDW.

The Eppendorfs that contain the anionic + natural fraction were taken into the speed vac. To obtain the cationic fraction, the extracts were passed through an anionic exchange column, Dowex 2% cross-linking 100−200 mesh in the nitrate form, and washed with DDW. The samples for the HSQC studies were prepared as described above.

To obtain extracts with reduced levels of GSH, the cells were incubated in a medium contains 250 μM BSO for 24 h 37 °C.

Quantification of Thiols. Thiols were quantified using the Ellman reagent. First, 20 μL of the sample were added to 75 μL of dilution buffer (30 mM Tris HCl, 3 mM EDTA pH = 8.2) and 25 μL of DTNB reagent (Ellman's reagent) in 400 μL methanol. The absorbance was measured at 412 nm using methanol as reference. The measurements were performed with Ultraspec 2100 p10 UV/visible spectrophotometer and 18/Q/10 700 μL quartz cuvetts. The thiol concentrations were determined from calibrations curves of NAC and GSH.

ICP-MS Analysis. The samples for total platinum determination by ICP-MS were prepared by adding to each of the lyophilized extracts 247.5 μL of water and 2.5 μL of 2.5 mM of cisplatin to give a total reaction volume of 250 μL (25 μM cisplatin in the resulting sample) and incubated for 12 h. The solvent was removed using a speed vac. The samples were then digested overnight by adding 100 μL of concentrated nitric acid (Subboiled suprapur 65% nitric acid, Merck) to each of the vials containing the dried samples. Prior to ICP-MS analysis the samples were diluted 200 times with 0.65% nitric acid and 0.1% hydrochloric acid.

All samples were analyzed using a Perkin-Elmer Sciex Elan 6000 DRC-e ICP-MS instrument (Perkin-Elmer, Norwalk, CT) equipped with a water cooled cyclonic spray chamber maintained at 4 °C and a micromist nebulizer (both from Glass Expansion, Melbourne, Australia). The sample uptake rate was 0.2 mL/min. Three Pt isotopes (m/z = 192, 194, and 195) were measured. The instrument sensitivity was approximately 14000 cps/ppb Pt, and the detection limit was estimated to 0.01 μg/L Pt. The accuracy of the method was tested by analysis of a serum reference material (ClinCheck: control, lyophilized serum, trace elements, Recipe, Germany) with a Pt content of 8.9 μg/L (control range 7.1−11 μg/L) and an average value of 9.6 μg/L was found.

Quantification was performed applying external calibration curves for the Pt-194 and Pt-195 isotopes. As a control for matrix effects, standard addition curves were obtained for 10% of the samples, but no significant difference between the slopes of the external standard curve and the standard addition curves were observed, indicating that no correction for matrix effects was needed.

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