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New extracellular resistance mechanism for cisplatin

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Abstract

The HSQC NMR spectrum of ^{15}N-cisplatin in cell growth media shows resonances corresponding to the monocarbonato complex, *cis*-$[Pt(NH_3)_2(CO_3)Cl]^-$, **4**, and the dicarbonato complex, *cis*-$[Pt(NH_3)_2(CO_3)_2]^{-2}$, **5**, in addition to cisplatin itself, *cis*-$[Pt(NH_3)_2Cl_2]$, **1**. The presence of Jurkat cells reduces the amount of detectable carbonato species by (2.8 ± 0.7) fmol per cell and has little effect on species **1**. Jurkat cells made resistant to cisplatin reduce the amount of detectable carbonato species by (7.9 ± 5.6) fmol per cell and also reduce the amount of **1** by (3.4 ± 0.9) fmol per cell. The amount of detectable carbonato species is also reduced by addition of the drug to medium that has previously been in contact with normal Jurkat cells (cells removed); the reduction is greater when drug is added to medium previously in contact with resistant Jurkat cells (cells removed). This shows that the platinum species are modified by a cell-produced substance that is released to the medium. Since the modified species have been shown not to enter or bind to cells, and since resistant cells modify more than non-resistant cells, the modification constitutes a new extracellular mechanism for cisplatin resistance which merits further attention.
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Keywords: Cisplatin; Resistance; Extracellular; HSQC NMR

1. Introduction

cis-Diamminedichloroplatinum(II), cisplatin (**1**; Fig. 1), has been used for decades for the treatment of neck, testicular, and other types of cancer [1–3]. A serious shortcoming with this and other drugs for treating cancer is that the tumor ultimately becomes resistant to the agent, forcing the discontinuance of chemotherapy. In the case of cisplatin, resistance mechanisms involving interception of the drug by cellular thiols, repair of platinated genomic DNA, rapid efflux/reduced uptake, and other processes are believed to be responsible for the resistance developed in cisplatin therapy [4–10]. A common feature of all known resistance mechanisms is that they are associated with the cell itself and not the medium outside the cell.

Earlier we used a sensitive technique, heteronuclear single quantum coherence NMR (HSQC NMR) and ^{15}N-labeled cisplatin to study the effects of Jurkat cells on the speciation of platinum in the culture medium outside the cell [11,12]. We found that the platinum species, *cis*-$[Pt(NH_3)_2(CO_3)Cl]^-$, **4**, Fig. 1, which forms in culture medium from the monoaquo/hydroxo species **2/3**, is modified by Jurkat cells in a manner which depends on the number of cells present in the medium. In subsequent studies [13] we showed that each Jurkat cell can modify 2.8 fmol of **4** within ~0.6 h and that the modified platinum does not bind to the cell. Because there is only a slow decrease $(-1.1 \pm 0.4\ \mu M\ h^{-1})$ in the amount of unmodified **4** remaining in the medium after 1 h, the cells subsequently lose their ability to modify **4**. We suggested that these results are consistent with a hitherto undocumented

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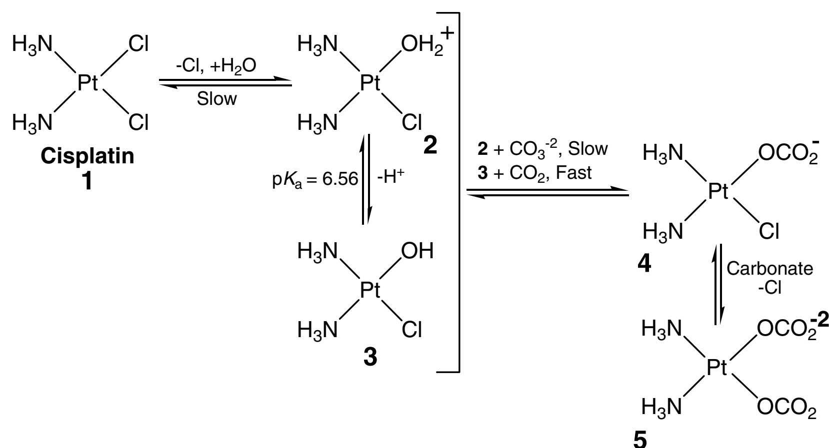

Fig. 1. Reactions of cisplatin in carbonate media.

extracellular detoxification mechanism in which the cells rapidly modify a portion of **4** in the culture medium. Subsequent work by us [14] with the anticancer drug carboplatin, [$Pt(NH_3)_2$(CBDCA-*O*,*O*′)], where CBDCA is cyclobutane-1,1-dicarboxylate, showed that Jurkat cells can affect the rate of disappearance of the HSQC NMR peak for carboplatin in a manner which is dependent on the number of cells present in the culture medium. Since the Jurkat cells take up only a small amount of the platinum present in the medium (<1%), the disappearance of the HSQC NMR peak of carboplatin cannot be due to absorption of the drug by the cells. The study suggested that the cells chemically modify carboplatin converting it into to an NMR-silent species.

Suspecting that these observations may indicate a new resistance mechanism for the platinum drugs that operates outside the cell, we made Jurkat cells resistant to cisplatin and used HSQC NMR to determine if and to what extent resistant cells can also affect the distribution of platinum species in the culture medium. In this report we show that resistant Jurkat cells are much more effective than normal Jurkat cells in modifying the monocarbonato complex **4** and the dicarbonato complex **5**, and that they can also modify cisplatin, **1**. The modifications take place even when the cells are removed from the medium, showing that the platinum-modifying substance is released by the cells to the medium so that the modification takes place outside the cell. These observations are consistent with a new extracellular resistance mechanism for cisplatin.

2. Materials and methods

2.1. Cisplatin resistant jurkat cells

Resistant Jurkat cells were prepared by exposing non-resistant Jurkat cells to increasing concentrations of cisplatin (Bedford Laboratories, Bedford, OH), in the concentration range, 1–50 μM, over the course of 5 months. The cells were maintained in suspension culture under a fully humidified atmosphere containing 5% CO_2 at 37 °C. The medium was RPMI-1640 supplemented with 10% (v/v) FBS, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 2.0 mM L-glutamine. For concentrations of cisplatin in the range, 1–10 μM, cells were exposed to drug for ~12 h, after which time, the cells were sedimented (200 × *g*) for 5 min, the drug-containing medium was removed and the cells were resuspended in fresh medium. These cells were allowed to recover for 3–4 days before exposure to a higher concentration of drug. For drug concentrations greater than 10μM, exposure of cells to drug was 2 h, followed by a 3–4 day recovery period before the next exposure to drug. Viabilities were assessed by light microscopy using a hemacytometer under standard trypan blue staining conditions [15].

2.2. ^1H–^{15}N HSQC NMR

The details of the ^1H–^{15}N HSQC NMR measurements involving ^{15}N-labeled were published previously [11]. Briefly described, the NMR experiments were two-dimensional, ^1H-^{15}N, with inverse detection and decoupling during acquisition without spinning the sample. Each NMR experiment involving 65 μM drug in the culture medium was 62 min (48 scans), giving 103 data points in the proton dimension and 64 t_1 values. The time for the first NMR time point ($t = 0$) was taken as the NMR data collection time plus ~15 min for temperature equilibration divided by 2 or ~0.63 h after the addition of labeled drug to the medium. The stock solution used in these experiments, which contained ~3 mM ^{15}N-labeled cisplatin in 154 mM NaCl, was allowed to reach equilibrium 24 h before addition of drug to the medium containing the cells. NMR data for a particular number of cells was collected as a function of time for the duration of the 10 h experiment, and plots of the concentrations of species present as a function of time constructed. The NMR experiments involving 400 μM drug were also 10 h in length. In these experiments, spectra were measured every 16 min for the first 4 h and every hour for

the remaining 6 h. For these experiments ~3 mM stock solutions of labeled drug in either 154 mM (normal saline) or 105 mM NaCl (chloride ion concentration of RPMI) were equilibrated for 24 h prior to their use.

The HSQC NMR spectra were collected in a capped tube at 37 °C using a Bruker DRX500 AVANCE spectrometer (^{15}N; 50.646 MHz) equipped with a 5-mm triple axis probe. The NMR chemical shifts were referenced externally to 1 M $(^{15}NH_4)_2SO_4$ in 95%, 5% H_2O/D_2O, which was acidified to pH 1 by addition of H_2SO_4. The ^1H chemical shifts were referenced to external $Me_3SiCD_2CD_2CO_2Na$, 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt, in a 23 mM, pH 7.2, bicarbonate solution. Volume integrations of peaks were obtained using Bruker software.

The samples for HSQC NMR measurement of the amounts of platinum species as a function of the number of resistant Jurkat cells present in the medium were prepared by suspending the desired number of cells in 900 μl of medium containing 95%/5% H_2O/D_2O, pH 7.2. Addition of 20 μl of the stock solution of ^{15}N-labeled cisplatin to the medium gave a final concentration of 65 μM total platinum in the medium in a final volume of 920 μl. The chloride concentration in the final solution, controlled by the RPMI 1640 in the medium, was 105 mM. No NMR peak for NH_3 *trans* to CO_3^{-2} of **4** was observed for greater than 5×10^5 resistant Jurkat cells in the NMR experiment.

Experiments showing that the platinum-modifying substance is released by the cells to the medium were done as follows: 1.0×10^7 resistant Jurkat cells were incubated in ~1.0 mL of medium for 1 h in a capped Eppendorf tube at 37 °C. After 1 h, the cells were sedimented ($200 \times g$) for 3 min and the 822 μL of medium containing the cell-released modifying substance was removed. To the recovered medium were added 50 μL of D_2O, and 128 μL of 3.13 mM ^{15}N-cisplatin previously equilibrated in either 154 or 105 mM NaCl to give a final concentration of total platinum of 400 μM in a final volume of 1 mL.

3. Results

3.1. Modification of species detected by HSQC NMR

To study the effects of resistant Jurkat cells on cisplatin, the HSQC NMR peak intensities of cisplatin, **1**, and its monocarbonato complex, **4**, I_1 and I_4, respectively, Fig. 2, were measured as functions of time after the addition of the drug to culture medium containing different numbers of cells. Fitting these plots of intensity vs. time to exponentials allowed for extrapolation back to $t = 0$ for each NMR experiment, giving the initial intensity of the species in the presence of the indicated number of cells. With no cells present in the culture medium, $I_1(0) + I_4(0)$ represents 65 μM drug, giving the conversion factor C between NMR peak intensity (peak volume) and concentration. As was earlier shown by us [12], the monocarbonato complex **4** has non-equivalent ammonia molecules, Fig. 1, and at neutral pH, the HSQC NMR peak for the ammonia molecule *trans* to Cl^- of **4** lies under the HSQC NMR peak for **1**. The concentration of **1**, for any number of cells, is then $C[I_1(0)–I_4(0)]$ and that of **4** is $C(2[I_4(0)])$. Initial concentrations obtained in this way are plotted against number of cells (diamonds for **1**, squares for **4**), and least-square fit to lines (Fig. 3). With no cells present in the culture medium the distribution of species in the 65 μM experiments is, $[\mathbf{1}] = (42.9 \pm 2.3)$ μM and $[\mathbf{4}] = (20.2 \pm 1.9)$ μM. The slopes are (-3.7 ± 1.0) μM per million resistant cells for **1** and (-8.6 ± 6.1) μM per million resistant cells for **4**. The same analysis earlier applied to normal Jurkat cells [13] gives slopes of

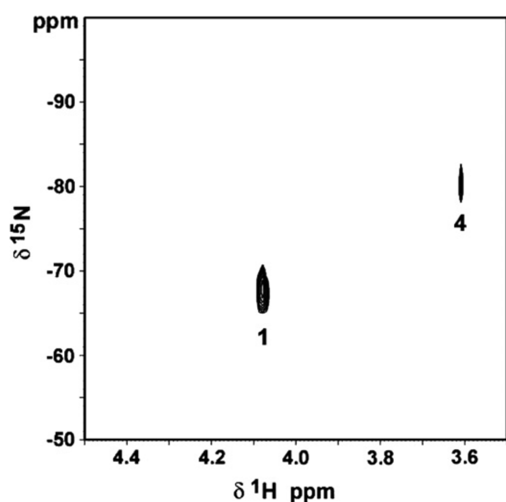

Fig. 2. $^1H–^{15}N$ HSQC NMR of 65 μM ^{15}N-cisplatin in the presence of 10^5 resistant Jurkat cells 1.6 h after the addition of the drug to the culture medium.

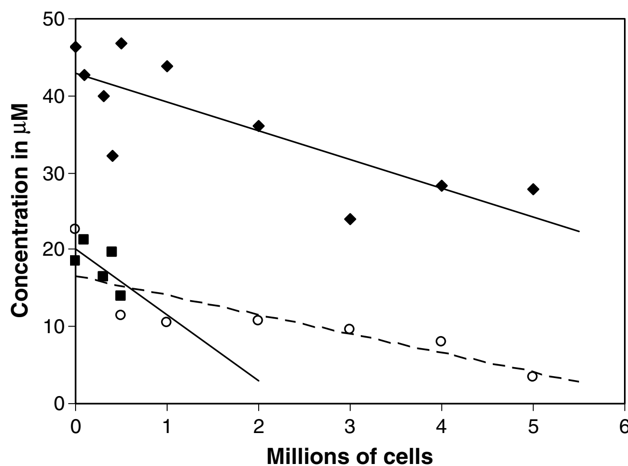

Fig. 3. The concentrations of **1** and **4** immediately after the addition of 65 μM cisplatin to the culture medium ($t = 0$), as functions of the numbers of resistant and normal Jurkat cells present in the medium. Black diamonds and black squares are for cisplatin, **1**, and the monocarbonato complex, **4**, respectively, in the presence of resistant Jurkat cells; open circles are for **4** in the presence of normal cells [13]. The concentrations of species present at $t = 0$ were as determined in the Materials and methods.

(1.0 ± 2.4) μM per million normal cells for **1** and (-2.5 ± 0.8) μM per million cells for **4**. The results for **4** with normal Jurkat cells are shown as circles and dashed line (Fig. 3).

In order to determine the number of moles of each platinum species that is modified by a single cell, we multiply the slopes by the reaction volume (920 μL) and divide by 10^6. We find that one resistant cell modifies (3.4 ± 0.9) fmol of **1** and (7.9 ± 5.6) fmol of **4** in less than ~0.6 h (the time at which we make our first NMR measurement). For comparison, a normal Jurkat cell modifies (2.8 ± 0.9) fmol of **4** and only slightly modifies **1**, [13].

3.2. Release of the platinum-modifying substance to the medium

As indicated in the Materials and methods, these experiments were done by placing normal or resistant Jurkat cells in fresh medium for 1 h, separating the cells from the medium by centrifugation, adding ^{15}N-cisplatin to the medium and collecting HSQC NMR data on the medium containing the drug. In order to decrease the time required to collect acceptable NMR data, the final concentration of total platinum in these experiments was 400 μM. In addition, stock drug solutions were pre-equilibrated at two different NaCl concentrations, 154 and 105 mM. Typical NMR results for these experiments are shown in Fig. 4, with a, b, and c for medium only, medium exposed to normal Jurkat cells, and medium exposed to resistant Jurkat cells. The true intensities for species **1** and **4** were obtained as I_1–I_4 and $2I_4$, respectively. The intensity of species **5** was never more than 10% of the intensity of **4**. Since **5**, a carbonato species like **4**, was also attacked by the modifying substance, I_5 was added to the intensity of **4** before obtaining the true intensities.

We expect that intensities for all species decrease with time because of the slow reaction of these compounds with substances in the medium [11,13,14], so it is not possible to obtain the conversion factor between intensities and concentrations. Therefore we have calculated the ratio of the true intensities for **1** and **4** which is the same as the concentration ratio [**1**]/[**4**]. For medium not exposed to cells, the true intensity for **1** is obtained by subtracting the intensity for **4** from that of **1** because the second resonance of **4** lies beneath the resonance of **1**. For medium exposed to cells, the changed pH shifts the second resonance of **4** so that it is seen directly and subtraction is not necessary.

If the conversion of **1** to **4** is fast relative to the rate of reaction of these compounds with substances present in culture media (amino acids, nutrients, etc.), this ratio is expected to decrease for the first few hours (the conversion of **1** to **4**) and then remain constant, assuming that **1** and **4** react at the same rate with substances in culture media. In Fig. 5 we plot this ratio vs. time for 400 μM cisplatin in (a) culture medium that has not been exposed to cells, (b) in medium exposed to normal Jurkat cells, and (c) in medium exposed to resistant Jurkat cells. The initial decrease in the ratio and its constancy for later times is very clearly shown. Each set of data was fitted to $A + Be^{-Ct}$ by minimizing the mean-square deviation, as shown. The value of the ratio at

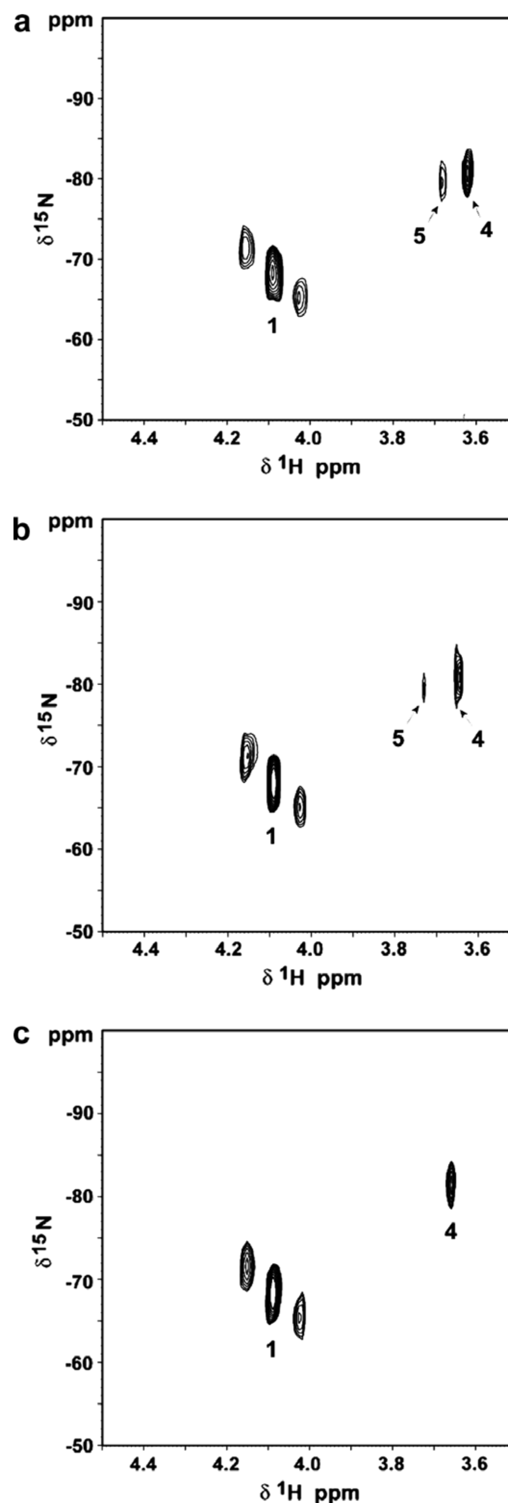

Fig. 4. HSQC NMR spectra of 400 μM ^{15}N-cisplatin in culture medium. (a) Drug in medium not exposed to cells. (b) Drug in medium previously exposed to 3×10^6 normal Jurkat cells for 1 h. (c) Drug in medium previously exposed to 3×10^6 resistant Jurkat cells for 1 h. Spectra taken 2.6 h after addition of drug to the culture medium. Species are as indicated in Fig. 1. The stock solution of drug used in these experiments was equilibrated in 154 mM NaCl.

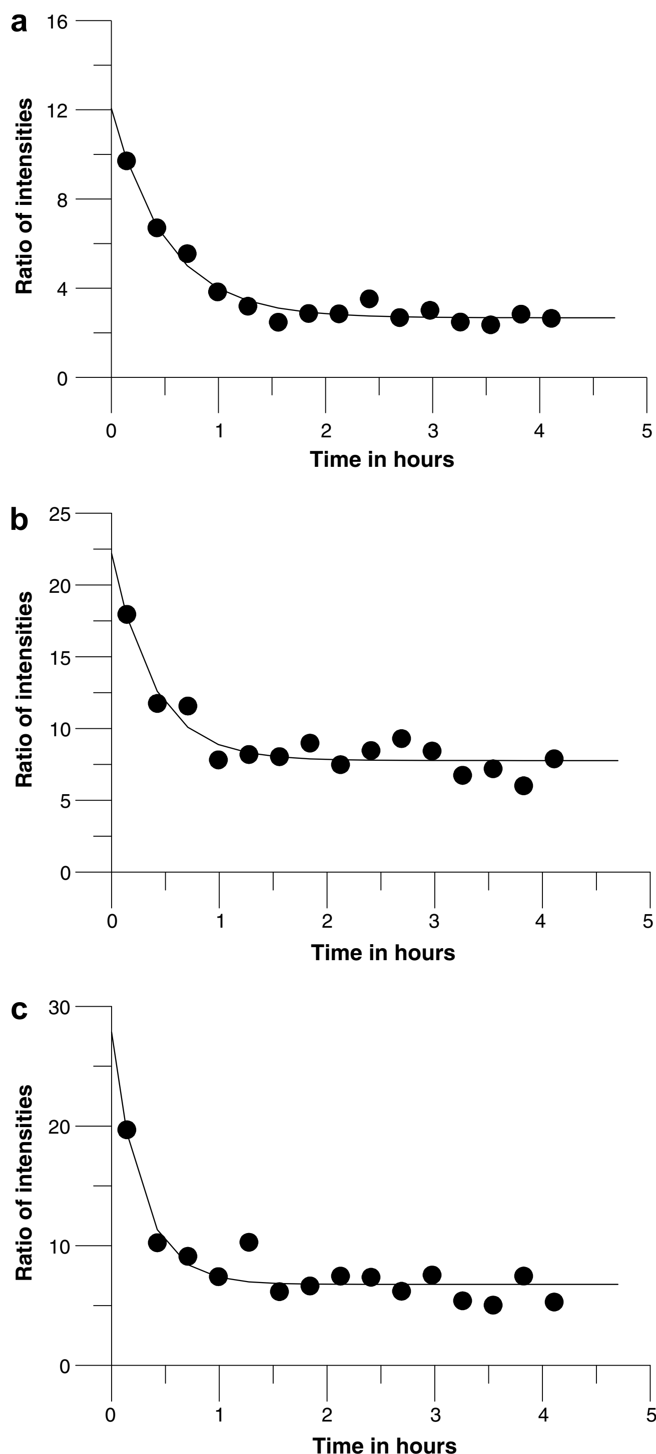

Fig. 5. Ratio of concentration of species **1** to concentration of species **4** in culture media from HSQC NMR, as function of time. Total concentration of drug, 400 μM, from a stock solution pre-equilibrated in 105 mM NaCl. (a) ^{15}N-cisplatin in culture medium not exposed to cells, (b) ^{15}N-cisplatin in medium previously exposed to normal Jurkat cells, (c) ^{15}N-cisplatin in medium previously exposed to resistant Jurkat cells. Each data set is fitted to $A + Be^{-Ct}$ by least-squares.

$t = 0$ (just after the reaction of cisplatin with substances in the medium) is A + B. For culture medium which has not been exposed to cells, the value is 12.1 ± 0.3, for culture medium previously exposed to normal cells, 22.2 ± 1.0 and for medium previously exposed to resistant cells 27.8 ± 1.3. Since the ratio [**1**]/[**4**] is greatest for resistant cells, they are more effective than normal cells in modifying **4** in the culture medium. While this same conclusion was reached in NMR experiments having the cells in contact with the drug (Fig. 3), Fig. 5 shows that the modifying substance is released by the cells and that the modification takes place in the medium.

4. Discussion

Fig. 3 shows the concentrations of cisplatin, **1**, and its monocarbonato complex, **4**, ~0.6 h after adding 65 μM cisplatin to suspensions containing culture medium and the indicated numbers of normal or resistant cells. As is shown in the plot, resistant Jurkat cells are much more effective in modifying **4** in the culture medium than are normal Jurkat cells. The linear fits shown have slopes of −3.7 and −8.6 μM per million resistant cells for species **1** and **4**; for normal cells, the slopes are ~0 (not shown – the actual value is $+1.0 \pm 2.4$ μM per million cells [13]) and −2.4 μM per million cells for species **1** and **4**. This means that a single normal cell modifies 2.8 fmol of **4**, and a single resistant Jurkat cell modifies 3.4 fmol of **1** and 7.9 fmol of **4**. Cisplatin, **1**, is more resistant to attack by the modifying substance in the medium than is **4**, whether the substance is produced by normal or resistant cells.

The conclusion that resistant cells can modify more **4** than normal cells is confirmed in the 400 μM experiments shown in Figs. 4 and 5. However, since the cells were no longer present when the NMR data were collected, it is clear that the platinum-modifying substance is released by the cells to the medium. It is also clear that this modifying reaction is rapid, occurring prior to the first NMR measurement, less than ~16 min. Because more **4** than **1** is modified by the cell-released substance, the platinum species re-equilibrate at early times, i.e., **1** converts to **4**, producing the exponential decay observed in Fig. 5. At later times, after the platinum species reach equilibrium with each other, the absolute intensities of both decrease slowly with time [11]. Since their ratio remains constant, they appear to react at equal rates with substances in the medium.

Although both the nature of the substance released by cells and the products formed are unknown, some explanations for the loss of the HSQC NMR signals can be ruled out. For example, while the platinum drugs readily react with thiols by ligand displacement, the initial products of these reactions, which have NH_3 *trans* to S^- are easily detected by HSQC NMR [13,16,17]. The fact that no HSQC NMR active product peaks are observed at ~−40 ppm (N), strongly suggests that attack by thiols, e.g. GSH, is not responsible for the modification of **1** and **4** in these studies. Another possibility for the loss of peak intensity is that the platinum compounds are binding to a macromolecule, which broadens NMR lines making

signals undetectable. However, earlier studies by Sadler and coworkers showed that HSQC NMR peaks for ^{15}N cisplatin are easily observed when the drug is bound to the 66 kD protein, human serum albumin [18]. It seems unlikely that the cell releases a high molecular weight protein for the modification of these platinum compounds. Furthermore, the amount of platinum compounds taken up by, or bound to, cells [13] is much less than the decrease in peak intensity seen in the HSQC NMR.

Most important, the modification of species **1** and **4** takes place in the absence of cells, so is an extracellular process. Since previous experiments [13] have shown that the modified species, which are HSQC NMR-silent, do not enter cells, this modification is a mechanism for drug resistance. Despite the fact that the molecular mechanisms of resistance to the platinum drugs have been studied for decades, inquiry in this area has largely focused in what happens *after* the drug enters the cell. However, recent studies suggest that tumors can recognize and reorganize their microenvironment to maximize their survival in the presence of anticancer drugs [18,19]. This ability, which has an important impact on the success of chemotherapy, could be the basis for an extracellular resistance mechanism that modifies toxins *before* they have the chance to penetrate the membrane and enter the cell.

In this report we use a sensitive NMR technique, HSQC NMR, to show that a substance released by Jurkat cells can modify cisplatin and its mono- and dicarbonato complexes in the culture medium. Since resistant Jurkat cells are able to modify more of the platinum compounds than normal cells, this appears to be an undiscovered resistance mechanism for cisplatin that operates outside the cell. In view of the fact that acquired resistance is one of the major limitations in platinum chemotherapy; this observation can have important consequences for the design of new platinum drugs for treating cancer. We hope that this report will stimulate interest in the role of carbonate in the molecular mechanism of action of the platinum drugs.

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